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VOL. XI — MAY, 1926 — No. 5

CONTENTS

LUTHER THOMPSON. The Blood-Agar Plate for Spore-Forming Anaërobes 305

E. B. FRED, W. H. PETERSON AND MAURICE MULVANIA. The Effect of Lactic Acid Bacteria on the Acetone-Butyl Alcohol Fermentation..... 323

ESTHER WAGNER STEARN AND ALLEN E. STEARN. Conditions and Reactions Defining Dye Bacteriostasis..... 345

HENRY N. JONES AND LOUIS E. WISE. Cellobiose as an Aid in the Differentiation of Members of the Colon-Aerogenes Group of Bacteria..... 359

C.-E. A. WINSLOW AND M. F. UPTON. The Electrophoretic Migration of Various Types of Vegetable Cells..... 367

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THE BLOOD-AGAR PLATE FOR SPORE-FORMING ANAËROBES

LUTHER THOMPSON

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The advantages of the blood-agar plate for the isolation and grouping of streptococci are well known. It now seems that the blood-agar plate may be equally useful in isolating and grouping another genus of bacteria, the spore-forming anaërobes. Under suitable conditions of anaërobiosis this medium has proved very satisfactory for securing growths, and the effect of these bacteria on hemoglobin resembles that obtained with streptococci, that is, some spore-forming anaërobes are hemolytic; some produce methemoglobin, and others have no effect on the red blood cells. It appears also that the plate method is more suitable for quickly securing pure cultures than the deep tube methods, so much used in the past, principally because the gas formed by many of these bacteria escapes from the thin layer of agar more readily than from the deep layer and thereby prevents mixing of the growths. Under similar physical conditions the anaërobic spore-formers should be as easy to secure in pure culture as, for example, the streptococci, although the literature gives the impression that pure cultures may be obtained only after long and tedious effort. More recently the Barber single cell method has been adopted by certain bacteriologists as the only reliable means of separating the anaërobes.

During the last year the blood-agar plate has been used as a routine for securing pure cultures of anaërobes at postmortem examinations with good results. I shall now present more particularly a tentative classification of pure cultures, obtained from

other sources, according to their effect on the blood-agar plate. Zeissler, used blood-agar-streak plates for growing *Clostridium welchii* and *Clostridium oedematis-maligni* and reports very characteristic surface colonies, stating that this method is most satisfactory for proving the purity of cultures. Richardson and Dozier speak of isolating delicate anaërobes on blood-agar plates, anaërobic conditions being obtained by the method of McIntosh and Fildes. They give no details as to cultures, but later Wagner, Dozier, and Meyer, using the same anaërobic method, describe the growth of *Clostridium botulinum*, *Clostridium sporogenes* and *Clostridium tetani* on poured blood-agar plates. Wheeler and Humphreys used blood-agar plates for growing anaërobes, securing anaërobic conditions in a jar exhausted with hydrogen. They were able to secure characteristic colonies of *Clostridium welchii*, *Clostridium botulinum*, *Clostridium sporogenes*, and *Clostridium histolyticum*, but found that *Clostridium tetani* and *Clostridium putrificum* grew poorly. Wagner reports that Pfenninger was unable to grow *Clostridium botulinum type C*, and the parabotulinus organism of Seddon on blood-agar plates. Hall has made extensive use of blood-agar slants for the cultivation of anaërobes, securing anaërobic conditions by Wright's method.

EXPERIMENTAL DATA

The medium used was hormone agar made after Huntoon's formula, with 1.5 per cent agar, and sterilized in flasks in amounts of 50 and 100 cc. The agar was melted and cooled to 45°C.

FIG. 1. CLOSTRIDIUM WELCHII (No. 2 HALL)
Double method, twenty-four hour culture. × 2

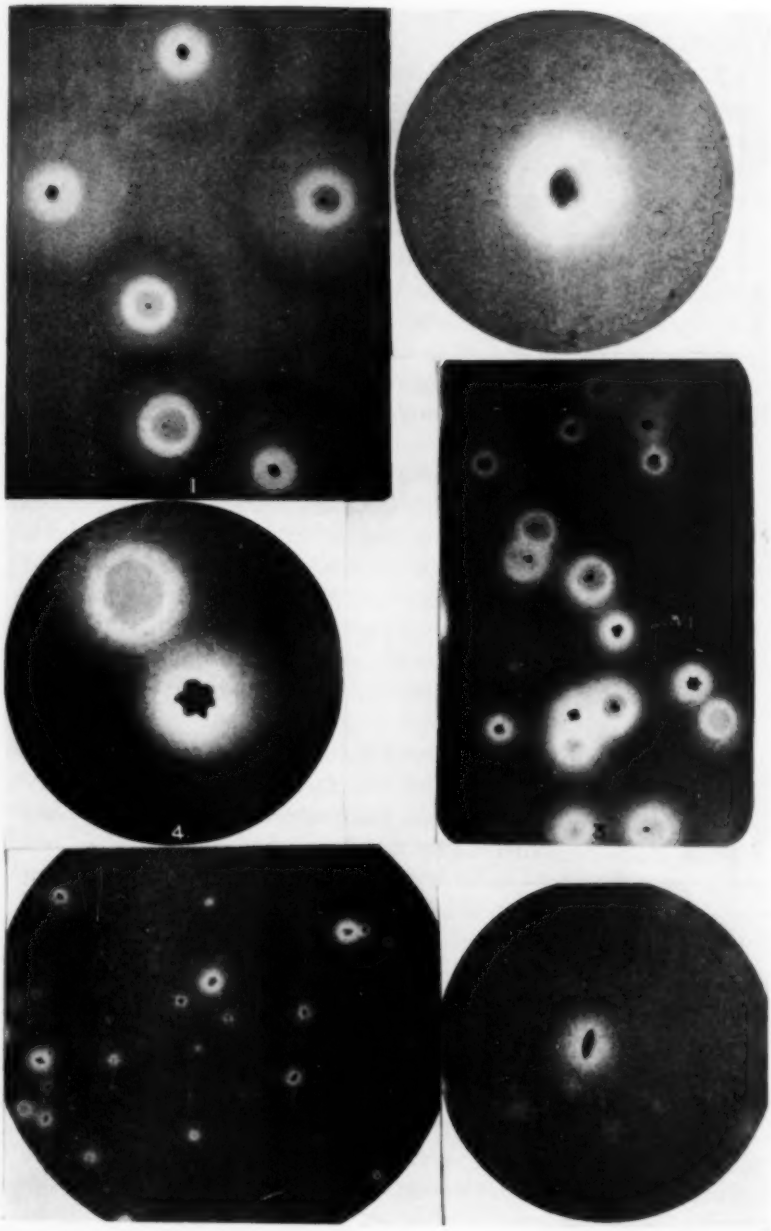
FIG. 2. CLOSTRIDIUM WELCHII (No. 2 HALL)
Double method, twenty-four hour culture. × 6

FIG. 3. CLOSTRIDIUM CENTROSPOROGENES (No. 76 HALL)
Double method, forty-eight hour culture. × 2

FIG. 4. CLOSTRIDIUM CENTROSPOROGENES (No. 76 HALL)
Double method, forty-eight hour culture. × 6

FIG. 5. CLOSTRIDIUM OEDEMATIS-MALIGNI (No. 18 HALL)
Double method, twenty-four hour culture. × 2

FIG. 6. CLOSTRIDIUM OEDEMATIS-MALIGNI (No. 18 HALL)
Double method, twenty-four hour culture. × 6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and 5 per cent human blood added; it was then dispensed into sterile tubes, 10 to 12 cc. to each tube. Serial inoculations were made from the material to be cultured, and plates poured. Anaërobic conditions were secured by means of a Novy jar exhausted with hydrogen, or by combining this with the alkaline pyrogallol method. In filling the jar with hydrogen the apparatus was so arranged that hydrogen entered the top of the jar while the air was drawn off from the bottom by means of a rubber tube attached to the glass tube in the stopper and long enough to reach to the bottom of the jar when the cover was in place. The ground-glass seals between the two halves of the jar and around the stopper were kept well coated with vaselin, and the jar, when in use, was held together with small clamps. A hydrogen cylinder fitted with a reducing valve was found to be the most convenient source of hydrogen. The hydrogen was allowed to run into the jar and the gas issuing from it was led into a pan of water by a tube with an inside diameter of between 4 and 5 mm. When the bubbles appeared at the rate of about 175 a minute it was assumed that the air could be largely removed from the jar in from twenty to thirty minutes, and if a tube of the gas was caught over water after this interval it would burn with very little explosion when ignited.

By this method the anaërobes which can tolerate small amounts of oxygen will grow well, but others grow poorly or only on very heavy inoculation. Repeating the process of exhaustion after half an hour to remove traces of oxygen which might diffuse out from the plates gave no better results. Therefore the remaining oxygen was absorbed in alkaline pyrogallate, which was done by placing an empty plate in the bottom of the jar to keep the others out of the liquid, and adding about 15 to 20 gm. of pyrogallic acid for a jar of approximately 3 liters capacity. It was necessary to have a jar whose diameter was somewhat larger than that of the plates. After exhausting with hydrogen about 50 cc. of 5 per cent sodium hydroxid was aspirated back into the bottom of the jar through the delivery tube, care being taken to turn the stopcock in the jar before air entered. It was found that cultures which grew poorly with the first method grew freely on the addi-

tion of the small amount of alkaline pyrogallate; it was therefore concluded that these cultures were inhibited by small amounts of oxygen. In some cases twenty-four hours is sufficient to secure well developed colonies, but for the majority forty-eight hours is preferable.

While the list of anaërobes studied by this method is not complete, enough of the more common ones have been examined to give a fairly good idea of the groups that may be expected. The difficulties attendant on securing authentic cultures for comparison make a complete study of all the described anaërobes a time-consuming project. Several platings were done on each culture, each being tried by both of the methods of anaërobiosis described. The following species have been studied, the numeral following each name indicating the number of strains: *Clostridium welchii* (10); *Clostridium centrosporogenes* (1); *Clostridium oedematis-maligni* (vibrion septique) (2); *Clostridium chauvei* (1); *Clostridium bifermentans* (2); *Clostridium histolyticum* (1); *Clostridium novyi* (1); *Clostridium tetani* (2); *Clostridium botulinum Type A* (3); *Clostridium botulinum Type B* (3); *Clostridium sporogenes* (1); *Clostridium tertium* (3) and *Clostridium putrificum* (1). The deep colonies were preferable to surface colonies for differentiation, since surface colonies are so largely influenced by moisture, and since in certain cases surface colonies are very few as compared with deep colonies. The following grouping is proposed, to serve, first, as a rapid laboratory method of indicating the more important pathogens which belong to the hemolytic group, and, second, as a guide to their more detailed identification.

BLOOD-AGAR GROUPING OF SPORULATING ANAËROBES

Group I. Hemolytic

A. Grow well in anaërobic jar exhausted with hydrogen (single method)[2]

1. Zone of hemolysis large compared to deep colony

a. Deep colony large, compact, often irregular, hemolysis clear

[2] For convenience the method of exhaustion with hydrogen is called the single method. When alkaline pyrogallate is also used it is called the double method.

(1) Zone of hemolysis surrounded by a deeper red zone

Clostridium welchii

(2) Zone of hemolysis not surrounded by a deeper red zone

Clostridium centrosporogenes

b. Deep colony small, compact, often lens-shaped

Clostridium oedematis-maligni (vibrion septique)

Clostridium chauvei

2. Zone of hemolysis small compared to size of deep colony

a. Deep colony filamentous on margin

Clostridium bifermentans

b. Deep colony compact and irregular

Clostridium histolyticum

B. Grow well only under strict anaërobic conditions (double method)

1. Zone of hemolysis large compared to size of deep colony

a. Deep colony compact, irregular, hemolysis clear

Clostridium novyi

b. Deep colony, filamentous on margin

Clostridium tetani

2. Zone of hemolysis small compared to size of deep colony

a. Deep colony compact, disc-shaped or irregular

Clostridium botulinum

b. Deep colony filamentous on margin

Clostridium sporogenes

Group II. Not hemolytic, producing green zone around colonies

A. Grow well in anaërobic jar exhausted with hydrogen

1. Deep colóny small, compact, lens-shaped

Clostridium tertium

FIG. 7. CLOSTRIDIUM CHAUVEI (NO. 6 HALL)
Double method, forty-eight hour culture. × 2

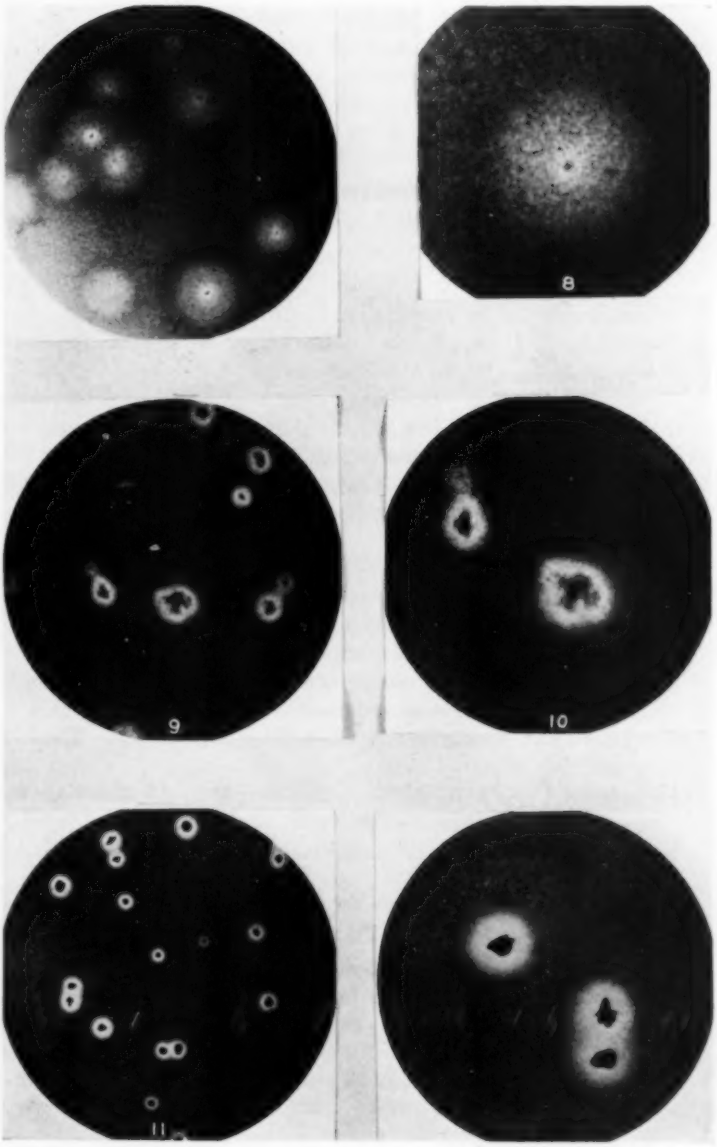
FIG. 8. CLOSTRIDIUM CHAUVEI (NO. 6 HALL)
Double method, forty-eight hour culture. × 6

FIG. 9. CLOSTRIDIUM BIFERMENTANS (NO. 102 HALL)
Double method, forty-eight hour culture. × 2

FIG. 10. CLOSTRIDIUM BIFERMENTANS (NO. 102 HALL)
Double method, forty-eight hour culture. × 6

FIG. 11. CLOSTRIDIUM HISTOLYTICUM (NO. 290 HALL)
Double method, forty-eight hour culture. × 2

FIG. 12. CLOSTRIDIUM HISTOLYTICUM (NO. 290 HALL)
Double method, forty-eight hour culture. × 6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Group III. Without effect on red blood cells
A. Grow only under strict anaërobic conditions
1. Deep colony punctiform
Clostridium putrificum

DESCRIPTION OF CULTURES STUDIED

Clostridium welchii

Culture 2. (I. C. Hall) Twenty-four hours' incubation
Good growth in twenty-four hours or less by both single and double methods
Surface colony: 2 to 3 mm., round, entire, convex
Deep colony: 1 to 1.5 mm., lens-shaped or irregularly lobate dense masses, with no filaments on margin (× 100)
Hemolysis: Marked. A primary clear zone surrounded by a wide diffuse zone, which is in turn bordered by a zone of darker red than the surrounding medium (figs. 1 and 2)
Morphology: Large rods of varying lengths, Gram-positive and Gram-negative. Rods often appear granular

Clostridium centrosporogenes

Culture 76. (I. C. Hall) Forty-eight hours' incubation
Grow well by both single and double methods, forty-eight hours required for well developed colonies
Surface colony: 1 to 2.5 mm., round, entire, or slightly irregular, some with raised centers, some with depressed centers
Deep colony: Dense irregular multilobulate masses, no filaments on margin (× 100)
Hemolysis: Clear zone whose diameter is 2 to 2.5 times that of the deep colony (figs. 3 and 4)
Morphology: Large rods, much like *Clostridium welchii* except larger

CLOSTRIDIUM OEDEMATIS-MALIGNI (VIBRION SEPTIQUE)

Culture 18. (I. C. Hall) Twenty-four hours' incubation
Grow well by both single and double methods
Surface colony: Variable in size and shape, some flat spreading films covering a quarter of the plate; some small (1 mm.) with root-like processes at margin, some small (0.5 mm.) round, entire, convex

Deep colony: Punctiform to 1 mm., disc-shaped or irregular, with knobs or fan-like projections, no filaments on margin (× 100)

Hemolysis: Some colonies show a small clear zone slightly larger than the colony itself, some a large diffuse zone three to five times the diameter of the colony (figs. 5 and 6)

Morphology: Some long rods, others medium or short, Gram-positive but not strongly so. Bipolar staining, and lightly staining barrel-shaped forms with or without polar granules common

Culture 421. Isolated September 11, 1924, at postmortem from a case of peritonitis. Cultural characters, morphology and pathogenicity for rabbits on intramuscular inoculations indicated *Clostridium oedematis-maligni*

On blood-agar plates it behaved in all respects like the preceding culture

Clostridium chauvei

Culture 6. (I. C. Hall) Forty-eight hours' incubation

Good growth by both single and double methods

Surface colony: 1 to 1.5 mm., round, entire, convex, slightly opaque

Deep colony: 0.2 mm., lens-shaped, compact, no filaments on margin (× 100)

Hemolysis: A wide zone compared to size of colony (2 to 3 mm.) Many red cells still intact giving a diffuse hazy appearance to the zone (figs. 7 and 8)

Morphology: Medium sized Gram-positive rods, irregular staining, swollen, round or clubbed forms common

Clostridium bifermentans

Culture 102. (I. C. Hall) Forty-eight hours' incubation

Good growth by both single and double methods

Surface colony: Some of small dew drop type, and others like a mat of filaments, rather dry looking

Deep colony: 0.25 to 1 mm., often showing a dense core set in a net work of filaments which contains coarse granules (× 100)

Hemolysis: The zone is not clear cut and extends but little beyond the line of growth (figs. 9 and 10)

Morphology: Large rods, Gram-positive and some partly decolorized. Granular forms common as in *Clostridium welchii*

Culture 507. Isolated at postmortem November 1, 1924, from the blood and spleen of a case of carcinoma of the ovary. The

results on coagulated egg, gelatin, brain broth, blood serum, litmus milk, and sugar broths indicate *Clostridium bifermentans*. This culture is nonpathogenic for rabbits when given intravenously or intramuscularly. On blood-agar plates it grows like culture 102, producing similar deep colonies and the same type of hemolysis.

Clostridium histolyticum

Culture 290. (I. C. Hall) Forty-eight hours' incubation

Good growth by both single and double methods

Surface colony: Some 1 mm., round, entire, convex, white (resembling staphylococcus colony), others 3 mm. to 1 cm., flat, spreading, with finger-like projections

Deep colony: 0.5 mm., compact, lens-shaped, heart-shaped, or irregular, with clear-cut margins having no projecting filaments (× 100)

Hemolysis: Zone clear and sharply defined, its diameter being about twice that of the colony (figs. 11 and 12)

Morphology: Medium sized Gram-positive rods, some appear granular

Clostridium novyi

Culture 140. (I. C. Hall) Forty-eight hours' incubation

Growth poor by single method, but rapid by double method

Surface colony: 3 to 4 mm., round, flat, with raised center, margin torn and irregular, color yellowish

Deep colony: Dense irregular masses 0.5 to 1.0 mm. with few short projecting hair-like filaments (× 100)

FIG. 13. CLOSTRIDIUM NOVYI (NO. 140 HALL)
Double method, forty-eight hour culture. × 2

FIG. 14. CLOSTRIDIUM NOVYI (NO. 140 HALL)
Double method, forty-eight hour culture. × 6

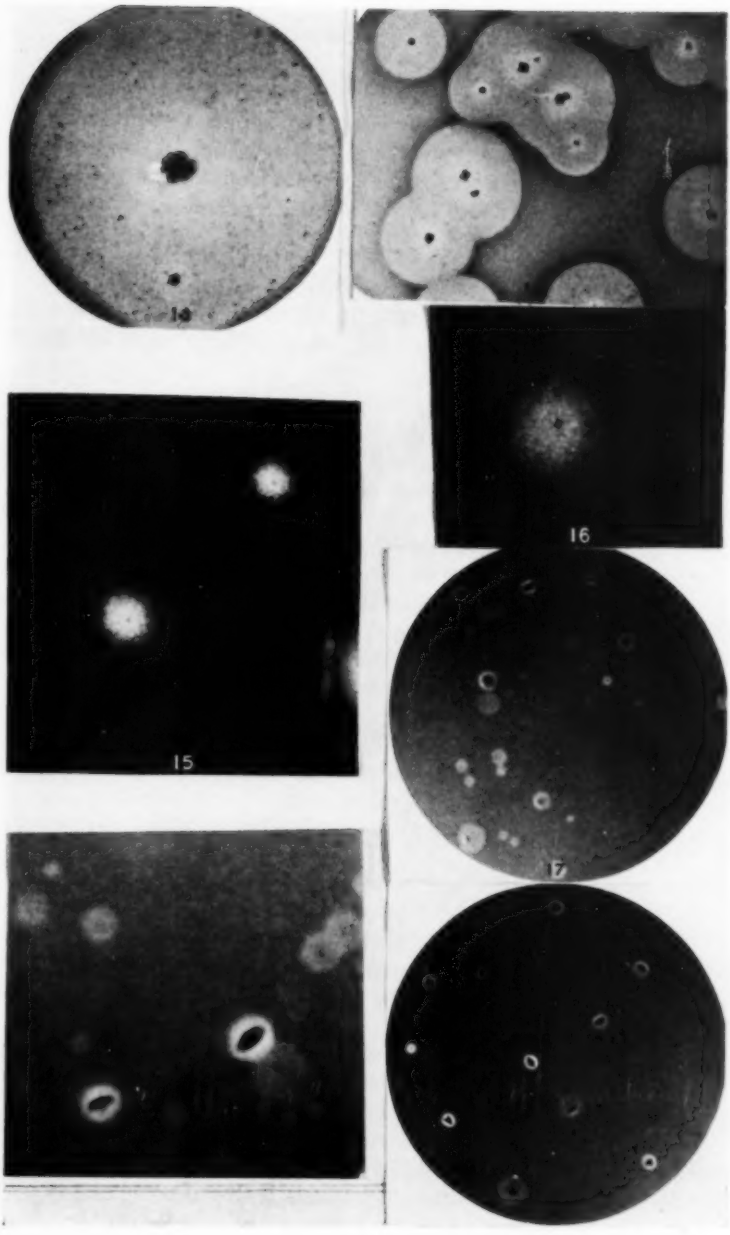
FIG. 15. CLOSTRIDIUM TETANI (NO. 1 HALL)
Double method, forty-eight hour culture. × 2

FIG. 16. CLOSTRIDIUM TETANI (NO. 1 HALL)
Double method, forty-eight hour culture. × 6

FIG. 17. CLOSTRIDIUM BOTULINUM TYPE A (NO. 9 PARK)
Double method, forty-eight hour culture. × 2

FIG. 18. CLOSTRIDIUM BOTULINUM TYPE A (NO. 9 PARK)
Double method, forty-eight hour culture. × 6

FIG. 19. CLOSTRIDIUM BOTULINUM TYPE B (NO. 178 HALL)
Double method, forty-eight hour culture. × 2



Hemolysis: Zones very large and clear, eight to ten times greater in diameter than the deep colony. There is often a darker red ring just surrounding the hemolytic zone (figs. 13 and 14)

Morphology: Large rods, some curved. Occasional shadow forms, or swollen forms with bipolar staining. Gram-positive

Clostridium tetani

Culture 1. (I. C. Hall) Forty-eight hours' incubation

Growth poor by single method, sometimes a few colonies develop after very heavy inoculation. Growth very rapid by double method

Surface colony: Flat, dry, irregular growths composed of matted filaments

Deep colony: 0.25 to 1 mm. Larger colonies very filamentous (× 100) with large granules among the filaments

Hemolysis: Zone large (eight to ten times greater in diameter than the colony), and hazy, without a clear-cut margin (figs. 15 and 16)

Morphology: Large rods and filaments, Gram-positive

Culture Research. (W. H. Park) Forty-eight hours' incubation

On blood-agar plates this culture grows and appears in all respects like culture 1

Clostridium botulinum Type A

Culture 9 Orr. (W. H. Park) Forty-eight hours' incubation

Growth occurs by double method only

Surface colony: Some are spreading, leaflike or rhizoid, others circular, entire, convex

Deep colony: Some heavy disc-shaped with entire margins 0.5 to 1 mm. Some small to punctiform, irregular, dense masses with or without a few filaments from margin (× 100). The colony shapes of all cultures of *Clostridium botulinum* are more variable than those of any other species studied (figs. 17 and 18)

Hemolysis: The larger disc-shaped colonies have a relatively narrow zone of hemolysis compared to the size of the colony, while the small colonies have a comparatively wide zone. The hemolysis is clear and the zone has a definite margin

Culture Memphis (W. G. McCoy) Forty-eight hours' incubation

Like the preceding culture it produces both spreading and convex

surface colonies, and also disc-shaped and punctiform deep colonies

Culture 372. (I. C. Hall) Forty-eight hours' incubation

The same varieties of deep colony as previously described, also certain ones resembling *Clostridium bifermentans*. The hemolysis is similar in all, that is, with relatively narrow clear zones

Clostridium botulinum Type B

Culture Dixon (W. H. Park) Forty-eight hours' incubation

Culture Nevin (W. G. McCoy) Forty-eight hours' incubation

Culture 178 (I. C. Hall) Forty-eight hours' incubation (See figures 19 and 20 of Culture 178)

What has been said of the type A cultures applies equally well to the type B cultures. The principal types of colony found in all botulinus cultures may be summarized as follows:

Surface: (1) spreading and (2) round, convex colonies

Deep: (1) disc, or lens-shaped colonies with or without coarse projections (fig. 20), and (2) punctiform or small irregular colonies

It was thought at first that these different varieties might indicate a mixed culture; consequently the different types were carefully picked to start new cultures. When these cultures were plated, the same variations occurred as in the original culture. As a further check these cultures obtained by picking different well isolated colonies were tested for toxicity. In all, nine subcultures representing the different colony types to be found in two type A and two type B strains were proved to be toxic for guinea pigs on feeding, and no nontoxic strains were found. This seems to indicate that *Clostridium botulinum* is quite variable as to colony form. The hemolysis, however, is a constant factor.

Clostridium sporogenes

Culture 90. (I. C. Hall) Forty-eight hours' incubation

Growth poor by the single method, but abundant by the double method

Surface colony: 0.25 to 1 cm., fern-like or arborescent, some flat, lobate or ameboid

Deep colony: A dense core 0.2 to 0.5 mm., round or slightly irregular, surrounded by a radiating network of tangled filaments (× 100)

Hemolysis: A clear sharply defined zone 1.5 to 2 mm. in diameter (figs. 21 and 22)

Morphology: Medium-sized rods, Gram-positive

Clostridium tertium

Culture 518. (I. C. Hall) Twenty-four hours' incubation

Growth occurs by either single or double method, but seems to be better if anaërobiosis is not too complete. Incubation for a longer period than twenty-four hours is unnecessary in the cultures studied

Surface colony: Most are round, 0.5 to 1 mm. convex, entire, and slightly milky in color. Some are flat, spreading, 0.5 to 2 cm. with irregular lobate margin

Deep colony: Oval, disc-like, compact bodies, 0.5 mm. without filaments on the margin (× 100)

Hemolysis: Negative. If plates are incubated by the single method there is a narrow faint green zone surrounding the colonies, more marked after the plates have been removed from the jar for an hour or two and the blood has regained its bright red color. *Clostridium tertium* is a representative of the group of anaërobes producing green zones on blood-agar plates (figs. 23 and 24)

Morphology: Long slender rods, usually partly decolorized by gram stain

Culture 441. Isolated September 19, 1924, at postmortem, from a case of gangrene of the retroperitoneum. This culture corresponds

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FIG. 20. CLOSTRIDIUM BOTULINUM TYPE B (NO. 178 HALL)
Double method, forty-eight hour culture. × 6

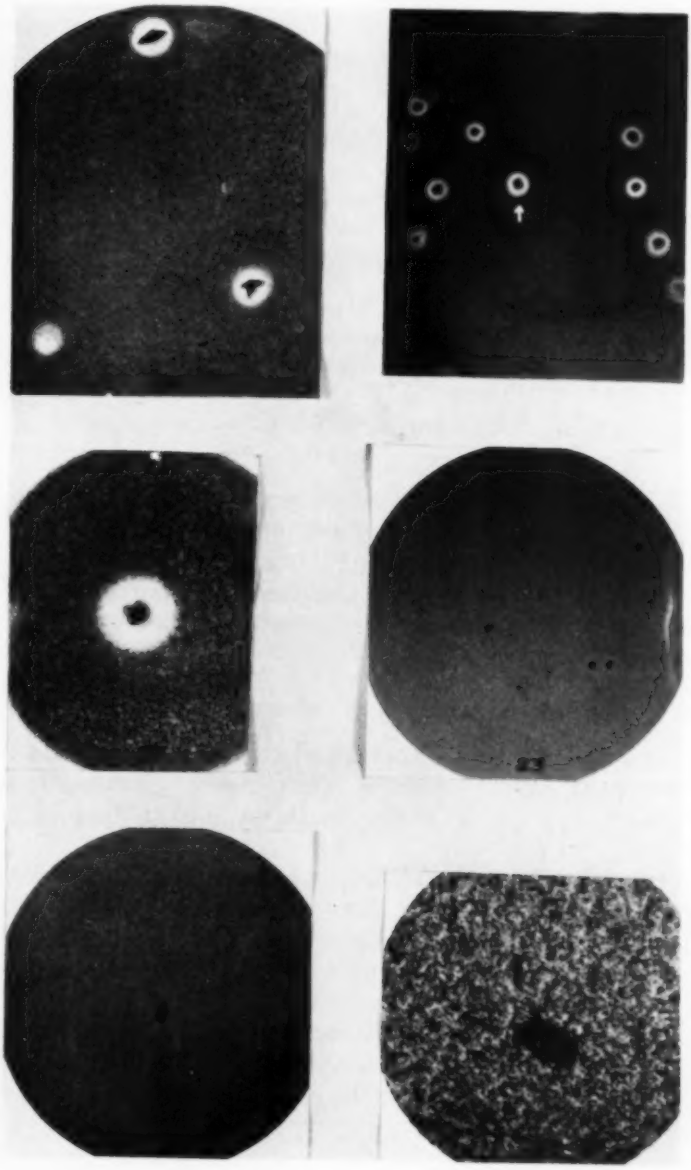
FIG. 21. CLOSTRIDIUM SPOROGENES (NO. 90 HALL)
Double method, forty-eight culture. × 2

FIG. 22. CLOSTRIDIUM SPOROGENES (NO. 90 HALL)
Double method, forty-eight hour culture. × 6

FIG. 23. CLOSTRIDIUM TERTIUM (NO. 518 HALL)
Single method, twenty-four hour culture. × 2

FIG. 24. CLOSTRIDIUM TERTIUM (NO. 518 HALL)
Single method, twenty-four hour culture. × 6

FIG. 25. CLOSTRIDIUM PUTRIFICUM (NO. 38 HALL)
Double method, six-day culture. × 20



morphologically and culturally with *Clostridium tertium*, and gives the same effect on blood-agar plates as the preceding culture

Culture 542–2. Isolated November 23, 1924, at postmortem from the lung in a case of terminal bronchopneumonia. All cultural characters are like those given above. In addition to these strains of *Clostridium tertium* other anaërobes producing green zones on blood agar have been isolated. As yet they have not been identified with any species described at present

Clostridium putrificum

Culture 38. (I. C. Hall) Six days' incubation
Growth was slow and was obtained only by the double method
Surface colony: None
Deep colony: Very tiny lens-shaped or oval compact masses
Hemolysis: None (fig. 25)

This culture grows very slowly in any medium. It is a representative of the nonhemolytic group. Other members of this group have been encountered in routine anaërobic culturing, but thus far they have not been identified with any described species. It is hoped that in time enough of them may be collected to make possible a comparative study.

SUMMARY AND CONCLUSIONS

The anaërobic blood-agar plate, when used as described, offers a very good general means of isolating and cultivating spore-forming anaërobes. It is also an aid in judging the purity of cultures.

Three groups of the anaërobes can be made, classified according to their effect on red blood cells: (1) the hemolytic group, comprising all of the well known pathogenic forms investigated in this work; (2) the group producing methemoglobin, or green zone colonies, and (3) that without effect on hemoglobin.

The blood-agar plate offers a rapid method of judging the significance of spore-forming anaërobes which may be encountered in clinical bacteriology, since the common pathogenic forms are hemolytic.

As an additional cultural method for identification of anaërobes, the blood-agar plate should be as valuable, if not more valuable, than any one of the test media commonly employed, such as brain broth, coagulated egg broth, and litmus milk.

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THE EFFECT OF LACTIC ACID BACTERIA ON THE ACETONE-BUTYL ALCOHOL FERMENTATION[1]

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In the manufacture of solvents by the fermentation process, Thaysen (1921) and Speakman and Phillips (1924) have shown that lactic acid bacteria often cause serious losses. The antagonism between *Granulobacter pectinovorum*[2] and the lactic acid bacteria is usually so marked that within twelve to eighteen hours after inoculation the growth of the acetone-butyl alcohol organism is suppressed.

In previous papers (Fred et al., 1925, and Stiles et al., 1925) the occurrence and the fermentation characteristics of some of these harmful lactic acid bacteria from corn mash have been discussed.

According to their effect on the acetone-butyl alcohol fermentation, these lactic acid bacteria may be divided into three groups: first, long rod forms usually granulated and very injurious to the butyl alcohol organism, second, long and short rod forms which may or may not be granulated, but never with large distinct granules as seen in group one, and which are also harmful to the butyl alcohol organism, but not so injurious as the preceding group; third, rod forms often very small, almost cocci, which are harmless or only slightly injurious to the growth of the butyl alcohol organism. These lactic acid organisms may be divided in other ways according to their fermentation products. For example, nos. 2, 3, 4, 7, 15, 19, 20 and 21, in table 1, produce

[1] Published with the Approval of the Director of the Agricultural Experiment Station.

[2] For convenience the term Granulobacter or Granulobacter pectinovorum is used throughout this paper in place of Bacillus granulobacter pectinovorum.

large amounts of lactic acid and very little CO_2 in the breaking down of sugars, while nos. 5, 6, 8, 9, 10, 11, 12, 13, 14, 16, 17 and 18 produce small amounts of acid, mannitol from fructose, ethyl alcohol from aldo-hexoses, and considerable amounts of CO_2 from these sugars.

The present communication deals with the harmful effect of the lactic acid bacteria on the butyl alcohol organism, and also with some of the factors that favor the growth of these lactic acid bacteria in mashes. Two phases of the subject have received special study; the persistence of the various lactic acid bacteria in mashes, and the nature of the agent injurious to the butyl alcohol-producing organism.

EXPERIMENTAL

The cultures of the lactic acid bacteria used in this study were isolated from mash of contaminated fermenters of the acetone-butyl alcohol fermentation. From more than two hundred cultures obtained from mash, fifteen of the more distinctive strains were selected for special study. In addition to the organisms from corn mash, five cultures of lactic acid bacteria isolated from other sources were used. Through the kindness of the Curator of the Lister Institute, London, transfers of the original culture of *Bacterium volutans*, Fleming and Thaysen, were supplied. Although this culture had been kept in the laboratory for several years, it had not lost its ability to injure *Granulobacter pectinovorum*. This culture from the Lister Institute will be discussed under the name, *Bacterium volutans*.

Some of the cultures have been under study for almost three years, and during this time have never shown a permanent loss in toxicity.[3] Occasionally the lactic acid bacteria develop in mash without producing any harmful effect on the growth of *Granulobacter pectinovorum*. Subsequent transfers from this same culture, however, have always shown that this apparent loss of toxicity is only temporary. The so-called non-toxic

[3] The term toxicity as used in this paper relates to the injurious effect of lactic acid bacteria on the butyl-alcohol organism and not to any specific substance elaborated by the former.

strains of the granulated lactic acid organism have not been encountered, although variations in degree of toxicity are not uncommon. If transfers are not made at frequent intervals, many of these high acid-forming bacteria are soon lost. Attempts to carry these cultures on such media as peptone agar or beef peptone agar, with or without sugar, proved unsatisfactory. The best results were obtained from cultures grown on potassium phosphate glucose yeast-water agar.

Effect of lactic acid bacteria on the growth of Granulobacter pectinovorum in corn mash

The inhibiting effect of the lactic acid bacteria on the growth of *Granulobacter pectinovorum* is quickly and easily determined from corn mash cultures inoculated with these two organisms. In a corn meal mash, the growth of *Granulobacter pectinovorum* may easily be detected by its amylolytic activity, as indicated by the decrease in viscosity, and opaqueness of the medium. In addition to these changes, the growth of the butyl alcohol organism results in a vigorous gas production, and also a compact head consisting of protein, fibrous material and slime. This head is forced to the top of the liquid in the early stages of the fermentation, and remains as a thick compact mass. The lactic acid bacteria on the other hand produce very little gas and no head. In the presence of certain strains of lactic acid bacteria, the characteristic gas and head of the butyl alcohol fermentation are lacking, and most of the mash remains as an unfermented residue in the bottom of the flask.

To measure the antagonism between the lactic acid and butyl alcohol bacteria, small Erlenmeyer flasks containing 100 cc. of 8 per cent corn mash were inoculated with 1 cc. of a twenty-four-hour old culture of *Granulobacter pectinovorum* and an equal quantity of a lactic acid culture. The gas and head formation were noted and at the end of three days the titratable acid and pH value determined. The results are shown in table 1. From the figures of this table it is clear that many of the lactic acid bacteria inhibit markedly the growth of the butyl alcohol organism, while others exert very little effect. Those forms commonly found in

water infusions of cereals, such as *L. leichmanni*, *L. mannitopoeum*, *Bact. volutans*, and *L. gracile* are especially harmful to the growth of *Granulobacter pectinovorum*. An exception to this is noted with

TABLE 1

Showing the effect of lactic acid bacteria on the growth of Granulobacter pectinovorum and on the acid produced in corn mash

Eight per cent corn mash

NUMBER	CULTURE NAME OR NUMBER	GROWTH OF GRANULOBACTER IN THE PRESENCE OF VARIOUS STRAINS OF LACTIC ACID BACTERIA		REACTION AFTER THREE DAYS	
		Growth	Head	0.1 N acid in 10 cc. of culture	H ion
				cc.	*pH*
1	Control*	Good	Good	3.5	5.4
2	*L. leichmanni*	Poor	None	12.2	3.6
3	*L. leichmanni*	Poor	None	11.9	3.6
4	*L. leichmanni*	Poor	None	11.3	3.7
5	*L. mannitopoeum*	Poor	None	10.9	3.7
6	*L. mannitopoeum*	Poor	None	10.0	3.8
7	*Bact. volutans*†	Poor	None	10.0	3.7
8	*L. mannitopoeum*	Poor	None	9.7	3.7
9	*L. mannitopoeum*	Poor	None	9.0	3.8
10	*L. gracile*	Poor	None	9.0	3.8
11	*L. gracile*	Poor	None	8.8	3.8
12	*L. intermedium*	Fair	Fair	7.9	3.8
13	*L. intermedium*	Fair	Fair	5.6	3.9
14	Culture 19	Fair	Good	4.7	5.0
15	Culture 60	Good	Good	4.7	5.2
16	Culture 7	Good	Good	4.5	5.2
17	Culture 4	Good	Good	3.8	5.2
18	*L. pentoaceticus*	Good	Good	3.8	5.2
19	*Streptococcus lactis*	Good	Good	3.7	5.5
20	*L. bulgaricus*	Good	Good	3.5	5.4
21	*L. acidophilus*	Good	Good	3.4	5.4

* *Granulobacter pectinovorum* alone.

† *Bacterium volutans* probably identical with *L. leichmanni*.

L. pentoaceticus and cultures nos. 4, 7 and 19. Culture no. 60, a streptococcus, has no effect on the growth of *Granulobacter pectinovorum*. The results suggest that the inhibition of the solvent-forming organism is related to the amount of acid produced.

Relation between the size of inoculum and the amount of acid formed

Five hundred cc. portions of corn mash in Erlenmeyer flasks were inoculated with an active *Granulobacter pectinovorum* culture and also with varying amounts of the lactic acid organism. At regular intervals samples were drawn from these flasks and the reaction measured.

TABLE 2

Effect of Lactobacillus leichmanni on acid production in corn mash inoculated with Granulobacter pectinovorum

NUMBER	AGE	GRANULOBACTER ALONE		GRANULOBACTER PLUS 1 CC. OF L. LEICHMANNI		GRANULOBACTER PLUS 5 CC. OF L. LEICHMANNI		GRANULOBACTER PLUS 10 CC. OF L. LEICHMANNI	
		Acid*	pH	Acid*	pH	Acid*	pH	Acid*	pH
		cc.		cc.		cc.		cc.	
1	After Beginning	0.2	6.6	0.2	6.6	0.3	6.5	0.3	6.4
2	3 hours	0.4	6.0	0.5	5.6	0.6	5.4	0.8	5.0
3	6 hours	0.8	5.3	1.3	4.8	1.3	4.6	1.5	4.4
4	9 hours	2.0	5.0	2.4	4.8	2.4	4.4	2.3	4.4
5	12 hours	3.5	4.8	4.1	4.4	3.9	4.0	3.1	4.0
6	15 hours	3.9	4.6	4.8	4.4	4.1	4.0	4.2	4.0
7	18 hours	4.2	4.4	5.1	4.2	4.5	3.8	4.4	3.8
8	21 hours	2.9	4.2	7.6	3.7	4.7	3.6	4.9	3.6
9	24 hours	2.5	4.6	8.4	3.6	5.0	3.6	5.0	3.6
10	27 hours	2.2	4.8	9.0	3.6	5.2	3.6	5.1	3.6
11	30 hours	2.0	4.8	9.3	3.4	5.8	3.6	5.4	3.6
12	3 days	3.2		11.4		10.0		9.8	
13	7 days	4.1		13.0		10.8		10.4	
14	14 days					11.7		11.4	
15	30 days	4.4		13.5		11.7		11.4	

* 0.1 N acid in 10 cc. of culture.

The formation of titratable acid and change in hydrogen ion concentration during the fermentation are given in table 2. Within three hours after inoculation, the flasks which received lactic acid bacteria showed a great increase in the hydrogen ion concentration. This gain in active acidity was especially noticeable in the cultures which received the largest amounts of lactic acid culture. By the end of six hours this rapid increase in the dissociated acid reached a pH of 4.8 or below while the

control containing *Granulobacter pectinovorum* alone did not exceed pH 5.3. The low buffer content of corn mash and consequent high dissociation of acids in all probability soon renders the mash unsuitable for the growth of the solvent-forming organism. The highest titratable acid occurred where small rather than large inocula of the lactic acid bacteria were used. High production of titratable acid is the result of a fine balance between the growth of the acetone-butyl alcohol organism and the lactic acid bacteria in the early stages of fermentation. If one or the other

TABLE 3

Relation between age of lactic acid cultures and their inhibiting effect on Granulobacter pectinovorum

In 5 per cent corn mash

NUMBER	ORIGINAL CULTURE	KIND OF GRANULOBACTER FERMENTATION				
		Age in days of the original culture added to corn mash				
		2	7	14	21	42
1	*Granulobacter* plus *L. leichmanni*	None	Poor	Poor	Fair	Good
2	*Granulobacter* plus *L. leichmanni*	None	Poor	Fair	Fair	Good
3	*Granulobacter* plus *L. intermedium*	Poor	Fair	Fair	Fair	Good
4	*Granulobacter* plus *L. intermedium*	Poor	Fair	Fair	Fair	Good
5	*Granulobacter* plus *L. mannitopoeum*	Poor	None	None	None	None
6	*Granulobacter* plus *L. mannitopoeum*	Poor	None	None	None	None

completely dominates the fermentation, then it is impossible to secure a high acid production. The maximum acid production was obtained with a 1 per cent inoculum of *Granulobacter pectinovorum* and a 0.2 per cent of *L. leichmanni*. In other words, to secure a high total acidity of the mash, the combined action of the two organisms is required. These data will be discussed in connection with another experiment.

Although the total acidity was measured after three, seven, fourteen and thirty days no marked gain in acid was found after the third day. The two cultures which received the larger

amounts of *L. leichmanni* did show a decided increase in acid after the thirtieth hour.

Attempts to favor the growth of one of these groups of organisms, and thus overcome the other, by incubating at various temperatures was tested repeatedly. Without exception, the lactic acid organisms soon dominated the fermentation.

The persistence of lactic acid bacteria in corn mash

Laboratory studies have shown that in the usual culture media many of the lactic acid bacteria, especially the high acid formers, exist for only a short time. Whether or not this same condition obtains in such natural substrates as corn mash is not known. It seems probable that these organisms will persist for a much longer time in cereal mashes. To secure an answer to this question a large number of tests with various strains of the lactic acid bacteria, and with various media, have been carried out. Only a summary of the more important points will be presented.

Flasks containing 300 cc. of a 5 per cent corn mash were inoculated with different strains of the lactic acid bacteria, *Lactobacillus leichmanni*, *Lactobacillus intermedium*, and *Lactobacillus mannitopoeum*. Twelve hours later, 30 cc. of the acetone-butyl alcohol organism were added to each flask. *L. leichmanni* is typical of the high acid, non-mannitol-forming, rod-shaped, granulated lactic acid bacteria possessing decided toxic properties; the other two organisms are small rods, representative of the low acid, mannitol-forming bacteria, and are not so toxic to *Granulobacter pectinovorum*. At first all of the cultures showed gas production, especially the flasks inoculated with *L. intermedium* and *L. mannitopoeum*. At the end of twenty-four hours the flasks which received *L. leichmanni* ceased to show gas, and a large deposit of unfermented starch remained. The other cultures gave a better fermentation, and only a small amount of unfermented starch remained.

To test for the presence and the toxicity of these lactic acid bacteria, samples were drawn from the original stock flasks and seeded into tubes of sterilized corn mash, as follows: 1 cc. portions of these mixed cultures were added to duplicate tubes of

20 cc. each of corn mash, and twelve hours later 1 cc. of an active culture of the acetone-butyl alcohol organism was added.

When forty-eight hours old, enrichment cultures were made (that is, transfers to fresh tubes of mash), and an active culture of *Granulobacter pectinovorum* was added. The results of these enrichment cultures, which were carried out simply as confirmatory tests, to eliminate the possibility that the change in reaction due to the inoculum might be the cause of the injury to the butyl alcohol organism, are not recorded in the tables.

The results obtained from the associated growth of the two kinds of organisms in fresh mash are given in table 3. The horizontal columns on the right of this table ("kind of granulobacter fermentation") show the toxic effect of the lactic acid bacteria on the butyl alcohol fermentation. The terms used in this table, for example "none," mean that transfers from the original culture into fresh mash and then seeded with fresh cultures of Granulobacter failed to give any growth. For each test after two, seven, fourteen, twenty-one and forty-two days old, fresh vigorous cultures of the acetone-butyl alcohol organism were added.

The results show definitely that in the mashes of this experiment the toxicity of *L. leichmanni* decreases with an increase in age until, after twenty-one to forty-two days, no injury is noted. The *L. intermedium* culture failed to show any decided change in toxicity after the second day. Apparently this member of the lactic acid family is not very harmful to the growth of *Granulobacter pectinovorum*.

In relation to time, *L. mannitopoeum* behaves in a manner entirely different from the high acid former *L. leichmanni;* the toxicity increased rather than decreased in the older cultures. The formation of neutral substances, alcohol instead of lactic acid, probably accounts for the longevity of this culture in mash.

Additional evidence on the longevity of the lactic acid bacteria is given in table 4. Here the procedure differed from that given in the foregoing experiment. The amount of active culture of *Granulobacter pectinovorum* was reduced from 10 per cent to 2.0 per cent, and also the amount of the lactic acid inoculum was

greatly reduced. Instead of allowing a period of incubation, both cultures were added at the same time. This change in method of inoculation resulted in quite different fermentations. The butyl alcohol organism showed a most active fermentation for more than forty-eight hours, and but little starch was left undigested.

TABLE 4

Relation between age of and different treatments of lactic acid cultures and their inhibiting effect on Granulobacter pectinovorum

In 5 per cent corn mash

NUMBER	KIND AND AMOUNT OF INOCULUM		ORIGINAL FERMENTATION (HEAD)	REACTION AFTER 1 MONTH (0.1 N ACID IN 10 CC. OF CULTURE)		KIND OF GRANULOBACTER FERMENTATION		
						Age in days of the original lactic acid cultures added to corn mash		
	Organism	Per cent		cc.	pH	60	180	365
1	*Granulobacter* alone	2.00	Good	4.5	4.1	Good	Good	Good
2	*L. leichmanni* alone	0.02	None	1.3	4.4	None	None	None
3	*Granulobacter* plus *L. leichmanni*	2.00 0.02	Good	4.4	3.9	None	None	None
4	*L. intermedium* alone	0.02	None	0.8	4.4	Poor	Fair	Fair
5	*Granulobacter* plus *L. intermedium*	2.00 0.02	Good	3.8	4.2	Poor	Fair	Fair
6	*L. mannitopoeum* alone	0.02	None	0.6	4.5	None	Poor	None
7	*Granulobacter* plus *L. mannitopoeum*	2.00 0.02	Good	8.0	3.7	None	Poor	Poor

As shown by Peterson, Fred and Domogalla (1924), the *Granulobacter pectinovorum* fermentation increases the buffer content of the medium. This gain in buffer, which is caused by the increase in soluble nitrogenous compounds, and the production of acids of low dissociation, coupled with the decrease in fermentable sugar, should bring about conditions favorable to the persistence of the lactic acid bacteria. The results shown in Tables

3 and 4 support this statement. The original *Granulobacter pectinovorum* fermentations recorded in table 3 were stopped long before the carbohydrate was consumed, while in table 4 these fermentations continued until almost all of the carbohydrate was destroyed. This difference in the original *Granulobacter pectinovorum* fermentation no doubt accounts for the long persistence of the lactic acid bacteria in the mashes of table 4.

TABLE 5

The production of lactic acid in mixed cultures of Granulobacter pectinovorum and L. leichmanni

Calculated for 1 liter of mash

NUMBER	KIND AND AMOUNT OF INOCULUM		0.1 N ACID IN 10 CC.	SOLVENTS	ZINC LACTATE	
	Organism	Per cent			Weight	Water of crystallization*
			cc.	*grams*	*grams*	*per cent*
1	*Granulobacter* alone	1.0	3.3	12.7	0.0	
2	*Granulobacter* plus *L. leichmanni*	1.0 0.05	4.0	11.2	1.53	16.7
3	*Granulobacter* plus *L. leichmanni*	1.0 0.25	4.6	10.5	2.25	15.1
4	*Granulobacter* plus *L. leichmanni*	1.0 0.5	7.1	0.2	4.84	15.5
5	Pancreatin and *L. leichmanni*	1.0	6.3	0.0	2.97	13.0

* Theory for inactive lactic acid, 18.2 per cent; for the active form, 12.9 per cent.

The presence of the lactic acid bacteria in the old mash was traced by microscopic examinations. When the cultures were two hundred and three hundred sixty-five days old, isolation plates were poured and the organisms secured in this way compared with the original stock lactic acid cultures. No differences in fermentation reactions or toxicity between the old and the new subcultures were noted.

An experiment similar to the foregoing was carried out with the

stock cultures kept at 37°C. instead of room temperature, 20 to 22°C. Since it was assumed that the organisms would probably die much quicker at the higher temperature, samples were drawn every five days and tested for the presence of harmful organisms. The lactic acid bacteria were alive regardless of the medium in which the cultures had been kept, whether mash fermented with Granulobacter or unfermented mash. These tests were discontinued after thirty days.

Solvent and lactic acid production in mixed cultures of Granulobacter pectinovorum and L. leichmanni

In a mixed culture the rôle played by each microörganism can be followed chemically by determining the product most characteristic of each: for Granulobacter, the solvents, acetone, and butyl alcohol; for *L. leichmanni*, lactic acid. A fermentation in which Granulobacter predominates will be high in solvents and low in lactic acid, and the reverse will be true where *L. leichmanni* is the dominating factor. It must be borne in mind, however, that the lactic acid organism can not grow to any extent upon the unmodified corn mash, and a suitable medium for its development depends upon the amylolytic and proteolytic action of Granulobacter. By varying the amount of inoculum in a series of flasks, fermentations grading from an essentially Granulobacter to that of a chiefly *L. leichmanni* type may be secured. The data from such a series are given in table 5. In order to make certain that lactic acid was being measured, the zinc salt was prepared and its water of crystallization was determined. The data show that solvents and lactic acid are inversely proportional to one another. As the quantity of *L. leichmanni* used in the inoculum was increased, solvent production decreased and the amount of zinc lactate increased.

An interesting result of the associated action of these bacteria is the effect on the relative quantity of d and l lactic acid produced. As shown in a previous publication *L. leichmanni* produces in pure culture almost entirely levo-lactic acid. In the case of no. 5, which was a pure culture of *L. leichmanni*, the water of crystallization is 13.0 per cent and is very close to that for the

levo enantiomorph. In nos. 2, 3, and 4, which were mixed cultures of the two bacteria, the water of crystallization shows the production of a larger quantity of dextro-acid by *L. leichmanni* than in no. 5. The effect of association on the forms of lactic acid and other products will be dealt with more fully in a later publication.

The nature of the inhibiting agent

The product of the lactic acid-forming organism, responsible for the injurious effect on the solvent-forming bacteria, has not been definitely identified, although the subject has been studied and much discussed. In view of our knowledge of the harmful influence exerted by acid-producing organisms on their associates, it would seem that we should look first to acidity as the cause of this antibiotic relation.

Speakman (1920) showed that during a normal fermentation of corn mash by *Granulobacter pectinovorum* a typical curve of acidity may be traced, which consisted of three phases. There is observed a rapid rise in the production of acid to a maximum. This is followed by a decided fall to a minimum and finally a slow rise from the minimum. Reilley and others (1920) have shown that the acids accumulated during the first phase of the curve are chiefly acetic and butyric with evidence of a third unknown kind. Schmidt, Peterson and Fred (1924) have since proved leucic acid to be present. Thaysen (1921) pointed out that a "serious infection" greatly modifies the normal acid curve as to time and extent of rise and fall. Such modifications are accompanied by irregularities in the entire fermentation, showing usually a rise of acidity above the maximum. Reilley and his associates state in their publication, that such erratic curves of acidity are associated with large amounts of lactic acid. The bacteria most commonly found to be responsible for the changes here noted belong to the lactic-acid-producing group. In all the earlier discussions at hand concerning this interference with the work of *Granulobacter pectinovorum* wherever reference is made to the influence of contamination, it seems to have been taken for granted that the results were due to the acidity produced by the

foreign organisms. More recently, however, doubt has been expressed as to the validity of this belief. On the basis of work recently done, Speakman and Phillips (1924) contend that the effect can not be due to the acid produced by the lactic acid bacterium, and they adopt the tentative hypothesis that the inhibitory agent is a product of the nitrogen metabolism of the contaminating organism. Scarcity of experimental evidence on this point and lack of agreement as to the part played by the acid produced by the lactic acid bacteria, appeared to warrant a more intensive laboratory study of this problem.

Some factors that influence acid production in corn mash inoculated with the lactic acid bacteria alone

As reported by Speakman and Phillips (1924) all attempts to secure high acid production in corn mash alone inoculated with these contaminating forms of lactic acid bacteria failed. When glucose was used and Sorensen's phosphate added to the medium as a buffer substance the result was entirely different. Under the above condition the lactic acid bacteria form large amounts of titratable acid as shown in table 6. In the absence of *Granulobacter pectinovorum*, but in the presence of a buffer and of glucose, *L. leichmanni* produces a fairly high concentration of acid. The results show that the presence of a fermentable carbohydrate, like glucose, is not sufficient to bring about acid production in mash. It is essential that a buffer be present to take care of the free acidity. A similar result can be accomplished by pre-digesting the corn mash with pancreatin. A sufficient quantity of available carbohydrates and protein is thus produced and the buffer capacity of the medium is increased. In such a medium *L. leichmanni* produced a considerable quantity of lactic acid. From 500 cc.of culture, 1.47 gm. of zinc lactate with 13.04 per cent water of crystallization was obtained. Corn mash media are unsuitable for the growth of *L. leichmanni* in several respects: lack of fermentable sugar, low buffer capacity and possibly insufficient soluble protein and phosphates. If these deficiencies are corrected, no difficulty is experienced in bringing about growth of the organism and acid production.

TABLE 6

The formation of acid in corn mash inoculated with Lactobacillus leichmanni alone

4 per cent corn mash

NUMBER	TREATMENT	TITRATABLE ACID AFTER						
		At beginning	1 day	2 days	3 days	5 days	10 days	15 days
		cc.†	cc.	cc.	cc.	cc.	cc.	cc.
1	Mash alone	0.25	0.50	1.0	1.2	1.2	1.2	1.4
2	Mash plus 0.2 per cent phosphate*	0.70					3.0	3.2
3	Mash plus 2.0 per cent glucose	0.30	1.4	1.7	2.1	2.2	2.2	2.2
4	Mash plus 2.0 per cent glucose 0.2 per cent phosphate	0.75	3.6	5.2	6.2	6.6	6.8	7.4
5	Mash plus 2.0 per cent glucose 0.5 per cent phosphate	1.65	3.6	5.0	6.0	6.6	7.1	7.7
6	Mash plus 2.0 per cent glucose 1.0 per cent phosphate	2.35	3.2	4.8	6.0	7.4	7.5	8.2
7	Mash predigested with Takadiastase	0.40			1.0	1.1	1.7	
8	Mash predigested with pancreatin	0.50			5.9	6.3		

* Sorenson's phosphate $Na_2HPO_4 + 2\ H_2O$ was used.

† 0.1 N acid in 10 cc. of medium.

TABLE 7

The influence of the products produced by L. leichmanni in corn mash on the growth of the butyl-alcohol organism

5 per cent corn mash plus 1 per cent of glucose

NUMBER	AGE OF LACTIC ACID CULTURE	TREATMENT	GROWTH OF GRANULOBACTER IN MASH PREVIOUSLY INOCULATED WITH THE LACTIC ACID BACTERIA			REACTION
			Growth	Gas	Head	
	hours					pH
1	24	Sterilized	Good	Profuse	None	5.8
2	24	Neutralized and sterilized	Good	Profuse	Good	
3	48	Sterilized	Fair	Slight	None	4.7
4	48	Neutralized and sterilized	Good	Profuse	Good	
5	72	Sterilized	None	None	None	4.5
6	72	Neutralized and sterilized	Good	Profuse	Good	

The effect of heat and neutralization of acids on the fermentation of corn mash by Granulobacter pectinovorum

The following experiments were arranged with reference only to the rôle played by acids in the antagonistic effect on Granulobacter. A direct comparison was made of the true acidity produced by the lactic-acid bacteria and of the corresponding acidity produced by additions of pure acids to the culture medium. After sterilization, the lactic acid bacteria were inoculated into the flasks and allowed to grow for various lengths of time. At the end of the period specified, the cultures were heated sufficiently to kill the lactic acid organism. Five flasks of each group were then inoculated with a vigorous culture of *Granulobacter pectinovorum*, and development recorded as shown in the accompanying tables. A sixth flask in each group was reserved for the determination of titratable acidity, and the pH value produced by the bacteria.

The outline of this experiment and the results obtained are shown in table 7. While the lactic acid bacteria alone in corn mash plus glucose do not produce as high a titratable acid as is found in mixed cultures containing *Granulobacter pectinovorum*, the hydrogen-ion concentration is not greatly different. It is well-known that a pH value of 4.8 to 4.6 is unfavorable to *Granulobacter pectinovorum;* and that pure cultures of the lactic acid bacteria will, in glucose mash, produce a value even lower than these figures.

The effect of the acid produced by these lactic acid bacteria in preventing the growth of the *Granulobacter pectinovorum* is clearly shown from the figures of this table. If old mash cultures of the lactic acid bacteria are inoculated with Granulobacter, there is no sign of growth. However, when these same cultures are neutralized, the butyl-alcohol organism develops. This fact has been noted where *Granulobacter pectinovorum* had been dormant for as long as eight days. The mash culture was merely neutralized, and no new inoculation of Granulobacter made.

Additional data concerning the nature of the toxic agent were obtained from cultures which had been seeded with *L. leichmanni*

and *Granulobacter pectinovorum*. Within three days after inoculation these cultures reached a maximum acidity of 11.5 cc. of 0.1 N acid in 10 cc. These cultures were kept for two weeks, but did not show any gain in total acidity beyond that found on the third day. As found by transfers to fresh media, the lactic acid bacteria under the conditions of this test were entirely destroyed before the end of one week. The complete destruction of the lactic acid bacteria in these cultures offered an opportunity to measure the *Granulobacter pectinovorum* fermentation in highly toxic cultures in which the acidity may be neutralized but the cultures not subjected to the heat of sterilization. To carry out this test large flasks of the mash were neutralized, inoculated with a fresh culture of Granulobacter, and a representative sample removed for solvent analysis. The fermentation in this neutralized but unheated mash was not as vigorous as commonly noted in fresh mash. However, by the second day there was a strong gas production and well formed head. The analysis for solvents of this 6 per cent mash is shown below:

	Total solvents in 1000 cc. of culture gm.
At the end of second fermentation neutralized and reinoculated	8.88
At beginning of second fermentation	2.11
Gain	6.77

According to the conditions of this test, neutralization alone without heat removes the toxic agent from corn mash cultures. The evidence points strongly to acidity as the prime cause in the injury to the butyl-alcohol fermentation.

That the lactic acid radical is not only non-toxic to Granulobacter, but is actually destroyed by the organism, was proved by adding calcium lactate (0.12 to 0.24 per cent) to the corn mash before inoculation, and analyzing the culture at the end of the fermentation. Such cultures, if anything, gave a better head and gas production than the controls. A careful chemical analysis failed to show any lactic acid in the flasks containing the smaller amount of calcium lactate and only a trace in those to which the larger quantities had been added.

Effect of varying amounts of organic and inorganic acids.

In tables 8 and 9 are given the results of this study. The relation between hydrogen-ion concentration and growth of *Granulobacter pectinovorum* is the important point brought out by these

TABLE 8

The influence of varying amounts of organic acids on the growth of Granulobacter pectinovorum

In 5 per cent corn mash

NUMBER	KIND OF ACID	1 N ACID	REACTION	KIND OF GRANULOBACTER FERMENTATION		
				Growth	Gas	Head
		per cent	*pH*			
1	Lactic	0.2	5.7	Good	Profuse	Good
2	Lactic	0.4	5.6	Good	Profuse	Good
3	Lactic	0.6	5.6	Good	Profuse	Good
4	Lactic	0.8	5.1	Good	Profuse	Good
5	Lactic	1.0	4.8	Good	Medium	Fair
6	Lactic	1.2	4.7	Fair	Slight	None
7	Lactic	1.4	4.6	None	None	None
8	Lactic	1.6	4.3	None	None	None
9	Acetic	0.2	5.9	Good	Profuse	Good
10	Acetic	0.6	5.8	Good	Profuse	Good
11	Acetic	1.0	5.4	Good	Profuse	Good
12	Acetic	1.6	5.0	Good	Profuse	Good
13	Acetic	2.2	4.8	Fair	Medium	Good
14	Acetic	2.6	4.7	Fair	Slight	None
15	Acetic	3.0	4.6	Poor	None	None
16	Butyric	0.2	6.0	Good	Profuse	Good
17	Butyric	0.6	5.7	Good	Profuse	Good
18	Butyric	1.0	5.1	Good	Profuse	Good
19	Butyric	1.6	4.9	Good	Profuse	Good
20	Butyric	2.2	4.8	Fair	Medium	Fair
21	Butyric	2.6	4.7	Poor	Slight	None
22	Butyric	3.0	4.6	Poor	Slight	None
23	Butyric	3.2	4.5	None	None	None

tests. It is not the percentage of the acid but the pH value which determines its inhibiting effect on the butyl-alcohol fermentation. The mineral acids are even more effective in preventing growth of *Granulobacter pectinovorum* than the organic acids. This injurious effect varies slightly among the acids

tested. As seen in table 9 *Granulobacter pectinovorum* will grow in slightly lower pH with hydrochloric than with either sulfuric or phosphoric acids, but the difference is not great. Since *Granulobacter pectinovorum* has the power to destroy these organic acids, and thus reduce the hydrogen-ion concentration, it is to be expected that the organism will start at a slightly higher

TABLE 9

The influence of varying amounts of inorganic acids on the growth of Granulobacter pectinovorum

In 5 per cent corn mash

NUMBER	KIND OF ACID	1 N ACID	REACTION	KIND OF GRANULOBACTER FERMENTATION		
				Growth	Gas	Head
		per cent	*pH*			
1	Sulphuric	0.1	6.0	Good	Profuse	Good
2	Sulphuric	0.2	5.8	Good	Profuse	Good
3	Sulphuric	0.4	5.6	Fair	Medium	None
4	Sulphuric	0.6	5.2	None	None	None
5	Sulphuric	0.8	5.0	None	None	None
6	Hydrochloric	0.1	5.8	Good	Profuse	Good
7	Hydrochloric	0.2	5.6	Good	Profuse	Good
8	Hydrochloric	0.4	5.4	Good	Medium	None
9	Hydrochloric	0.6	5.2	Fair	Medium	None
10	Hydrochloric	0.8	5.0	Poor	None	None
11	Hydrochloric	1.0	4.8	None	None	None
12	Phosphoric	0.1	6.0	Good	Profuse	Good
13	Phosphoric	0.2	5.9	Good	Profuse	Good
14	Phosphoric	0.4	5.8	Good	Profuse	Good
15	Phosphoric	0.6	5.7	Good	Medium	Fair
16	Phosphoric	0.8	5.6	Fair	Slight	None
17	Phosphoric	1.0	5.4	Fair	Slight	None

acidity when this is due to organic acids, than when it is caused by unfermentable compounds.

The results here reported have reference only to the pH value necessary to prevent initiation of growth of the butyl-alcohol organism. Nothing has been ascertained as to the pH value which would check growth when once under way. It would no doubt be lower than that required to prevent the beginning of development, as Granulobacter is known to produce a very con-

siderable buffer effect when active in a medium. In mixed cultures, the development appears always to be in favor of the lactic acid-forming organism. In fact some hydrolysis of starch in the corn mash is necessary before the lactic acid organism has a suitable supply of carbohydrate upon which to act. During this initial growth, the Granulobacter would probably form the buffers mentioned.

The non-toxic effect of filtrates of cultures of L. leichmanni in the Granulobacter fermentation

The question has been asked, Is the antagonistic effect of the lactic acid bacteria due to non-acid substances which pass through a filter? To find an answer to the question, a great number of filtration experiments were carried out. Cultures of the lactic acid bacteria of various ages were passed through Berkefeld and Pasteur Chamberland filters of varying porosity. The bacteria were removed in this way and the neutralized filtrate added to corn mash plus *Granulobacter pectinovorum*. All attempts, however, to suppress the growth of the acetone-butyl-alcohol-forming organism with these filtrates failed.

Similar results were secured from experiments with collodion sacks. Here the different cultures were grown in the same medium separated only by the thin membrane. If it is assumed that the bacteria produce inhibiting substances which will diffuse through a membrane, then the harmful effect of the lactic acid cultures should be readily detected by the use of the special culture tube for collodion sacks (Mulvania, 1924). Here again, no injury other than that common to an acid reaction was ever noted.

SUMMARY

In the mash inoculated with *Granulobacter pectinovorum*, the common types of cereal lactic acid bacteria grow rapidly producing large amounts of lactic acid.

The formation of the lactic acid in the mashes is dependent upon the associated action of the two groups—the lactic acid and the butyl-alcohol organisms. The presence of the lactic acid

bacteria alone is not sufficient. The growth of *Granulobacter pectinovorum* favors the development of the lactic acid bacteria in several ways; by hydrolysis of the starch to fermentable carbohydrates, by proteolysis of the nitrogen compounds to amino acids—production of buffer substances. Although this association is beneficial to the lactic acid bacteria, it is extremely harmful to the solvent-forming organism.

These harmful lactic acid bacteria may be divided into various groups. The high acid-forming organisms described under the name *Lactobacillus leichmanni* are the most injurious to the butyl-alcohol fermentation. Next in order of their inhibiting effect, belong the organisms described as mannitol-formers, *Lactobacillus mannitopoeum*, *Lactobacillus gracile*, and *Lactobacillus intermedium*.

The persistence of the lactic acid bacteria in mash for long periods of time depends on the kind of organism and also on the degree of the associated growth with Granulobacter. The high acid-producing bacteria such as *L. leichmanni* persist for only a short time if they have had an abundance of fermentable sugar and available nitrogen. Quite the contrary is true of the mannitol-forming group of lactic acid bacteria; these usually produce much smaller amounts of acid and survive for a much longer period. In the mash alone, without *Granulobacter pectinovorum*, the lactic acid bacteria persist for at least one year, perhaps for a longer time. The presence of a protective colloid, starch, and the presence of only a small amount of fermentable sugar no doubt favor the longevity of the lactic acid bacteria. Strange to say, somewhat similar results in relation to longevity of lactic acid bacteria are obtained when the *Granulobacter pectinovorum* produces a very vigorous fermentation. Here also the conditions favorable to longevity of the lactic acid bacteria are present; namely, large amounts of buffer substances and relatively small amounts of acid. The life of the lactic acid bacteria is comparatively short, provided the growth of *Granulobacter pectinovorum* is sufficient only to hydrolyze much of the starch, but not to carry it through to the formation of neutral substances, and thus large amounts of acid are formed and only small amounts of buffer substances.

There is no evidence to indicate that lactic acid is more harmful to the growth of *Granulobacter pectinovorum* than the other organic or inorganic acids. When additions of pure acetic, lactic, butyric, sulphuric, hydrochloric and nitric acids are added to mash in amounts sufficient to give pH values of about 4.7 to 4.8, there is a well-defined inhibiting effect on the growth of the acetone-butyl alcohol organism. If this acidity is neutralized, the injurious property is removed. Similar results are obtained with mashes in which the acidity is produced by the lactic acid bacteria, provided the mashes are kept until all of the living lactic acid bacteria have died off. The evidence, taken as a whole, points to acidity as the chief factor injurious to the growth of *Granulobacter pectinovorum*.

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CONDITIONS AND REACTIONS DEFINING DYE BACTERIOSTASIS

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The continued study of bacterial cells from the point of view which assumes that they behave as conjugated proteins (Stearn and Stearn, 1924a and b) has led to an intensive study of the action of both basic and acid dyes in their bacteriostatic and staining reactions.

The use of buffers in bacteriological media is becoming more and more common, since it is generally conceded that bacteria have very decided pH limits of growth unless slow acclimatization beyond these limits has taken place. Little of the existing data on bacteriostatic agents has taken into account that the effective dilution of these agents varies greatly with the pH of the media, and since the pH fluctuates as growth proceeds it is absolutely necessary to have sufficient buffer present for its control.

Browning, Gulbransen and Kennaway (1920) found that the sterilizing effect of diamino-acridine methyl chloride is multiplied one hundred times by a change of pH from 4 to 11. There are, however, few known organisms which have natural pH limits of growth as wide as these authors suggest. In studying the pH effect on bacteriostatic action it is essential to remain well within the limits of growth, preferably within the optimum range of H-ion concentration.

I. J. Kligler (1918) in his study of bacteriostasis by dyes attempted to control the pH of the media by the addition of 0.5 per cent K_2HPO_4. This gives an initial pH of 7.1 and protects the media against formation of acids during growth, but has no buffering power against bases. Even then this is by no means

the mostly highly effective H-ion concentration for bacteriostatic action by basic dyes. According to Dernby (1921) there are organisms such as the staphylococcus, whose optimum H-ion concentration lies between the pH limits of 7.2 and 7.6. For such an organism our data here presented will show that basic dyes would be far more inhibitive at a pH of 7.6 than at 7.1—the former being incidentally the pH of human blood.

Traube (1912) found that Na_2CO_3 accentuates the toxicity of certain stains, notably crystal violet. Prowazek (1910) noted likewise that the addition of Na_2CO_3 increased the activity of methylene blue. Dernby and Davide (1923) state that eucupin and the quinine alkaloids are more effective against the staphylococcus and diphtheria bacilli in solutions which are more alkaline than the blood.

In the cases of acidic substances, Graham-Smith (1919) found quinine more effective in acid than in neutral or alkaline solutions. Davis and White (1918) found that acid chlor-mercury fluorescein was more active in acid solution.

Data showing to what extent it is important to determine the H-ion concentration at which a specific bacteriostat is most inhibitive for a specific organism are given below, which suggest answers to other relative and cogent questions. The pH range through which the effect of any dye was studied was not arbitrarily chosen but was the range through which the organism in question has been shown to grow. Except where otherwise stated these limits were obtained from Dernby (1921). In all cases the limits of the range were tested by growing the particular strains studied in 0.2 per cent lactose broth adjusted to the upper and lower limiting values of the particular range through which they were subsequently to be studied. The media were buffered by phosphate mixtures. A minimum total salt concentration of about one-fifteenth molar was required to hold the pH constant. This latter was checked both before and after incubation by the indicator method. Doubling the concentration of buffer material did not affect the results. In the cases where gentian violet was used a 0.2 per cent lactose broth was generally employed. It was found, however, that identical results were obtained when a 1 per cent glucose broth was used in its stead.

A. BASIC DYES

As Simon and Wood (1914) have suggested, we may assume that in the structure of the bacterial organism we find receptors for either acidic or basic substances. This idea might be represented by a type formula $R\frac{NH_2}{COOH}$, the acid group acting as receptor for a basic substance and vice versa; or it might be more simply represented by the formula HPrOH.

The present authors (1923) found that at a high pH range, from about 7.6 to 8.5, bacteria as they developed in gentian violet media would settle to the bottom as a deep purple precipitate, decolorizing the solution, whereas at a lower pH range, from 4.3 to 5, the bacteria which settled were only slightly stained and the solution remained a deep purple.

The following series of equilibria may be thought of as establishing themselves in a system of bacteria and dye. We will represent any basic dye by the formula DOH.

(1) $DOH \leftharpoons D^+ + OH^-$

(2) $HPrOH \rightleftharpoons H^+ + PrOH^-$

(3) $D^+ + PrOH^- \rightleftharpoons DPrOH$

(4) $DPrOH + H_2O \leftharpoons HPrOH + D^+ + OH^-$

Maximum bacteriostatic action occurs with maximum formation of the un-ionized dye-protein compound, DPrOH. A study of the above equilibria would lead, among others, to three predictions. Data bearing on each of these are presented below.

1. The long arrows in the above set of equations show the direction in which the equilibrium would shift for an increase in alkalinity. This direction is obvious for equations 1 and 2, and it is clear that the effect of hydroxyl ion concentration on number 3 is only indirect. Equation 4, the hydrolysis of the dye-protein compound, takes account of the fact that the dye will be much stronger as a base than the bacterial cell protein is as an acid, and thus for comparison of effect the dye is represented as ionized compared to the protein. Increase in alkalinity would aid, directly or indirectly, the formation of DPrOH, and thus

augment the bacteriostatic effect of a basic dye, in the equilibria represented by equations 2 and 4. It would work against the formation of DPrOH in equation 1. Since, however, the dye will be much more strongly ionized than the protein the effect of an increase in alkalinity on number 1 will be much less than on number 2,[1] and we should expect an increase in alkalinity to increase the effective dilution of the basic dye.

TABLE 1

BACILLUS DYSENTERIAE (SHIGA) LIMITING pH RANGE 6.2 TO 7.6					BACILLUS TYPHOSUS LIMITING pH RANGE 5.4 TO 9.1				
Gentian violet dilution	pH				Gentian violet dilution	pH			
	5.58	6.23	7.16	7.73		6.23	6.81	7.16	7.73
45,000	−	−	−	−	150,000	−	−	−	−
50,000	+	−	−	−	200,000	+	+	−	−
60,000	+	−	−	−	300,000	+	+	+	−
100,000	+	+	−	−	500,000	+	+	+	+
200,000	+	+	+	+	No dye	+	+	+	+

BACILLUS COLI LIMITING pH RANGE 4.4 TO 7.8					BACILLUS AEROGENES OPTIMUM pH 6.5*				
Gentian violet dilution	pH				Gentian violet dilution	pH			
	5.28	6.23	7.16	7.73		5.2	6.23	7.1	7.7
10,000	−	−	−	−	5,000	−	−	−	−
20,000	+	−	−	−	10,000	+	−	−	−
30,000	+	+	−	−	30,000	+	+	−	−
70,000	+	+	−	−	50,000	+	+	+	−
100,000	+	+	+	−	100,000	+	+	+	+
200,000	+	+	+	+					

* Smith (1922).

Table 1 gives the results of experiments with gentian violet on some Gram-negative organisms. Twenty-four hour agar

[1] An example will make this clear. If we take two weakly ionized substances, one weaker than the other, say with ionization constants 10^{-6} and 10^{-9}, and calculate the effect of a change of pH from 4.0 to 8.0, we find, assuming for simplicity that they are both bases, that the ion concentration of the stronger is decreased by 10 ten times while that of the latter is decreased by a million times. I.e., the same pH change affects the weaker one 10,000 times as much as the stronger in the above case, which is fairly typical.

cultures were washed off by the addition of sterile nutrient broth. To insure the same amount of inoculation two drops of bacterial suspension were transferred by means of a sterile pipette to each tube of 0.2 per cent lactose broth containing definite amounts of dye and buffered with a phosphate mixture. The results are those obtained after seventy-two hours incubation.

Table 2, giving results for strongly Gram-positive organisms, while in accord with the well known fact that this group of organisms is in general more sensitive to basic dyes than the Gram-negative shows clearly that the behavior toward these dyes under varying conditions is perfectly analogous to that of the Gram-negative organisms. The culture of staphylococcus used was isolated from an infected knee and was found to grow

TABLE 2

STAPHYLOCOCCUS AUREUS LIMITING pH RANGE 5.6 TO 8.1					STREPTOCOCCUS HEMOLYTICUS LIMITING pH RANGE 5.5 TO 8.0					
Gentian violet dilution	pH				Gentian violet dilution	pH				
	6.4	7.0	7.2	7.6		6.4	7.1	7.3	7.7	8.0
2,000,000	−	−	−	−	4,000,000	+	−	−	−	−
3,000,000	+	−	−	−	5,000,000	+	+	+	−	−
4,000,000	+	+	+	−	6,000,000	+	+	+	+	−
6,000,000	+	+	+	+	8,000,000	+	+	+	+	+
					10,000,000	+	+	+	+	+

in a concentration of gentian violet 1:2,000,000 at a pH of 6.4 even though that was beyond its optimum limits of growth. The growth limits are given as 5.6 to 8.1 but the optimum lies between 7.2 and 7.6.

Here also the data represent the results of seventy-two hours incubation.

Table 3 gives results obtained with two other basic dyes brilliant green, which is more strongly basic than gentian violet, and para-rosaniline, which is less strongly basic than gentian violet. The organism studied was *Bacillus coli*. Data for both forty-eight and seventy-two hours incubation are included. The peculiar behavior of the brilliant green should be especially noted. Its behavior was normal up to a pH of about 7, beyond

which its color faded out. Up to the point of this change it exerts an extremely powerful bacteriostatic action, but beyond this point this power is largely lost. The point is rather sharp since at 6.8 to 6.9 perfectly normal action is encountered while at 7.16 peculiar results are obtained.

Another striking phenomenon, which, unfortunately, it is impossible to show in our tables, is the *comparative quantity* of growth. In general it may be said that in such cases as repre-

TABLE 3

BRILLIANT GREEN					PARA-ROSANILINE				
Dye dilution	pH				Dye dilution	pH			
	4.95	5.28	6.46	7.16		5.28	6.23	7.16	7.73
50,000	−	−	−	−	5,000		(See note)		
	−	−	−	−					
75,000	−	−	−	−	7,500	+	+	−	−
	+	−	−	+		+	+	−	−
150,000	+	+	−	−	10,000	+	+	−	−
	+	+	−	+		+	+	−	−
300,000	+	+	+	−	15,000	+	+	+	+
	+	+	+	+		+	+	+	+
600,000	+	+	+	+					
	+	+	+	+					

Note: At this concentration there was growth in all tubes as evidenced by slight precipitation. This was very slight at the lower values of pH gradually increasing to the higher.

sented in table 1 for those concentrations of dye where there is growth in all tubes at all H-ion concentrations studied, the amount of growth falls off as the alkalinity increases, as one might expect from the remainder of the table. These "gradations" in quantity of growth with the pH are almost as striking as the "gradations" in the limiting dilution of dye with the pH, and furnish an equally striking substantiation of the prediction as to the pH effect.

II. Reversibility. The equilibria represented in equations 1 to

4 above should be reversible. As already pointed out (Stearn and Stearn, 1924b) the bacteria are probably not killed by these dyes except perhaps at fairly high concentrations, but are merely fixed and rendered temporarily powerless to multiply. The following is the evidence that this prediction is true.

Tubes containing dye in which no growth appeared after seventy-two hours incubation were treated with a solution containing the same concentration of dye but so acidified that the final pH would be lower than that at which the original incubation took place, and would be one at which decided growth had been shown to take place. Invariably, after twenty-four hours incubation, growth took place, shown not only by clouding of the media but also by streaking on agar and microscopically

TABLE 4

pH OF ORIGINAL BROTH (NO GROWTH OCCURRED)	APPROXIMATE pH OF ADJUSTED BROTH (GROWTH AFTER TWENTY-FOUR HOURS)	DYE DILUTION	ORGANISM
7.3	6.0	50,000*	*B. coli*
8.0	6.0	100,000*	*B. coli*
7.0	6.0	10,000*	*B. aerogenes*
7.7	6.0	7,500†	*B. coli*

* Gentian violet.
† Para-rosaniline.

examining the growth. This guaranteed that the cloudiness of the broth was due to the original organisms themselves which had again manifested their reproducing power, and not to the presence of a contaminating form. Experiments were made with both gentian violet and para-rosaniline. Table 4 includes a few of the many experiments performed along this line. In the case of the adjusted broth the control of the pH is not quite as close as in the other cases.

III. Basic strength of dye. A third prediction one might make is that the greater the basic strength of the dye the more effective a bacteriostat it will be at any one pH. This is brought out in equation 1, for the stronger the dye the greater the concentration of dye ion D^+. While no accurate data on the ionization constants of the dyes used are available, the effect on the basic or

acidic strength of the substitution of various groups in various positions in the molecule is more or less definitely known, and thus comparative basic strengths can be determined. (For a somewhat more full discussion of this question applied to the triphenylmethane dyes, see Stearn and Stearn, 1924b.) Table 5 compares the limiting dilutions of the basic dyes used on *Bacillus coli* at the same pH. The order is that of decreasing basic strength. It should be pointed out that from consideration of their formulas there is probably little difference between the basic strength of gentian violet and methyl violet. They should be nearly the same. The former has in it one more methyl group and might be a shade the stronger.

TABLE 5

DYE	LIMITING DILUTION		
	pH 5.2	pH 6.2	pH 7.7
Brilliant green	200,000	250,000	Power destroyed
Gentian violet	15,000	25,000	150,000
Methyl violet	10,000	15,000	70,000
Para-rosaniline	5,000	5,000	10,000

B. ACID DYES

A set of equilibria, analogous to those given for basic dyes, could be given for acid dyes. From such a set the three analogous predictions might be made. Data bearing on these points are presented below. These data are not as extensive as in the case of basic dyes but the general behavior is indicated almost as conclusively. Perhaps the most important feature regarding these data, one which has been mentioned but which should be emphasized, is that in this work the authors have limited their range of study to conditions of solutions in which the organisms are known to thrive in the absence of dye, so that the data are less equivocal than much of the material at present available in this field.

1. Increase in alkalinity with acid dyes should have the

opposite effect to that with basic dyes. Table 6 gives results for eosin, acid violet 5B, and acid fuchsin. Using a buffered 0.2 per cent lactose broth, a loopful of *Bacillus coli* suspension was inoculated and incubated for seventy-two hours. In the more alkaline solutions the dyes are seen to be less effective.

TABLE 6

DILUTION	pH						
	5.23	6.23	7.16	7.73	4.95	6.23	7.15
Acid fuchsin							
25	−			+			
50	−	−	+	+			
75	−	−	+	+			
100	−	+	+	+			
Acid violet							
25					−		+
50					−	−	+
100					−	+	+
200					+	+	+
Eosin							
25	−			+			
50	−	+	+	+			
75	−	+	+	+			
100	+	+	+	+			

TABLE 7

pH OF ORIGINAL BROTH (NO GROWTH OCCURRED)	pH OF ADJUSTED BROTH (GROWTH IN TWENTY-FOUR HOURS)	DYE DILUTION	ORGANISM
6.0	7.0	100*	*B. coli*
6.0	7.0	200*	*B. coli*

* Acid fuchsin.

II. Reversibility. As in the case of basic dyes, it was possible to take a tube in which no growth had occurred after seventy-two hours incubation, and, by adding alkaline dye solution, keeping the concentration of dye constant, to obtain vigorous growth in twenty-four hours. Table 7 is analogous to table 6.

III. Acidic strength of dye. Table 6 is arranged in the probable order of decreasing acidic strength of the dyes. A glance at these data will show the increase in bacteriostatic power with the acidic strength, keeping the pH constant.

C. EFFECT OF THE PREVIOUS ENVIRONMENT OF THE ORGANISM ON ITS SENSITIVITY TO DYES

The importance of this problem, from the point of view both of classification or identification and of resistance to bacteriostats and bacteriocides, is apparent. Below are presented the results of a few preliminary experiments in this direction. They will not be discussed at this time.

I. A twenty-four-hour growth of *Staphylococcus aureus* was stained for one minute with a sterilized solution of gentian violet by thoroughly mixing while still suspended on the agar slant. The stained organisms were then inoculated into nutrient broth, one tube at a pH of 6.2 and another at a pH of 7.7. After twenty-four hours incubation vigorous growth of the organism had developed in the broth at a pH of 6.2, but there was no growth whatsoever in the other tube.

II. For two weeks a culture of *Bacillus coli* grew in nutrient broth whose pH was kept at 6.2. Another culture was grown at a pH of 7.7. They were twice transferred to new media and kept at 37°. At the end of this period a loopful of the culture media was inoculated into methyl violet—0.2 per cent lactose broth. The results are included in table 8. Readings were made after twenty-four, forty-eight, and seventy-two hours. While the final readings were the same in the case of both cultures, the twenty-four-hour and even the forty-eight-hour readings were not. The culture which had been kept in the more alkaline medium at a pH of 7.7 is rendered temporarily more sensitive to the action of the methyl violet, and it is only after some time that it recovers its normal ability to multiply in the presence of the dye.

The results under "a" were from the culture kept for two weeks in a broth at a pH of 6.2, those under "b" were from the culture at 7.7 described above. The results under "c" came

from inoculations from a suspension prepared from an agar slant kept at a pH of about 7.0 for forty-eight hours.

TABLE 8

DILUTION OF METHYL VIOLET	pH											
	5.2			6.2			7.1			7.7		
	a	b	c	a	b	c	a	b	c	a	b	c
10,000	+	−	−	−	−	−	−	−	−	−	−	−
	+	+	−	−	−	−	−	−	−	−	−	−
	+	+	+	−	−	−	−	−	−	−	−	−
20,000	+	+	−	+	−	−	−	−	−	−	−	−
	+	+	+	+	−	−	−	−	−	−	−	−
	+	+	+	+	+	+	−	−	−	−	−	−
30,000	+	+	+	+	+	+	−	−	−	−	−	−
	+	+	+	+	+	+	−	−	−	−	−	−
	+	+	+	+	+	+	−	−	−	−	−	−
70,000	+	+	+	+	+	+	+	+	−	+	−	−
	+	+	+	+	+	+	+	+	−	+	+	−
	+	+	+	+	+	+	+	+	+	+	+	−
100,000	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+
200,000	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+

D. SUMMARY AND DISCUSSION

1. In defining the bacteriostatic strength of a dye it is necessary to state the pH at which it is to act.

2. A method of proving the reversibility of a dye bacteriostatic reaction has been shown. This indicates an added reason why it is essential to give repeated injections of gentian violet in its use intravenously.

3. For a series of organisms not too widely different in character, the same concentration of the same dye might easily be

given as the limiting inhibitive dilution merely by altering the reaction of the medium. This is brought out in table 9 using data obtained with gentian violet.

4. In several cases, with Gram positive organisms, unexpected results were obtained. For instance in certain series in which *Streptococcus hemolyticus*, isolated from the spinal fluid in a case of meningitis, was being studied, it was found that at a pH of 5.9 and in a concentration of gentian violet 1:2,000,000 growth occurred. Upon examination the culture proved to contain long chains of streptococci about half of which were Gram positive and the remainder distinctly and strongly Gram negative. Even after subculturing on agar chains of the cocci would exhibit this characteristic; i.e., a deeply stained blue coccus would be united to a deeply stained red one, etc. The growth

TABLE 9

ORGANISM	pH	
	Limiting dilution, 50,000	Limiting dilution, 100,000
Bacillus aerogenes	7.73	8 plus
Bacillus coli	7.16	7.73
Bacillus typhosus	6.81	7.16
Bacillus dysenteriae	Less than 5.0	Less than 5.0

in this particular tube seemed quite instructive and peculiar since no such thing had taken place at even higher dilutions of gentian violet and higher values of pH. Similar results were obtained with *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis*. Growth of any of these distinctly Gram positive organisms in scattered tubes of large series we found meant the presence of strains of so-called mutants which might develop other unlooked for characteristics. The study of these mutating strains is now in progress, and with it a study of the effective cause which stimulated this pronounced and peculiar action.

In conclusion it is a pleasure to acknowledge the suggestions of Dr. B. F. Sturdivant, Director of the Laboratories of the Pasadena Hospital, whose continued interest has been a source of inspiration.

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CELLOBIOSE AS AN AID IN THE DIFFERENTIATION OF MEMBERS OF THE COLON-AEROGENES GROUP OF BACTERIA[1]

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Contribution from the Bacteriological Laboratory of Syracuse University and the Department of Forest Chemistry of the New York State College of Forestry

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INTRODUCTION

The very general acceptance of the presence of colon bacilli in water supplies as an index of more or less recent fecal pollution has made the correct delimitation and ready identification of the species a matter of great importance.

More than thirty years ago, Theobald Smith (1890), pointed out that colon bacilli could be subdivided into two sub-groups according to their action upon sucrose. From that period to the present, many workers have interested themselves in the differentiation and classification of the species and subspecies of this group.

Of the twenty-two different species of colon-like organisms listed in the 1923 edition of Bergey's Manual, twelve produce acid and gas from sucrose while ten do not. On a statistical basis it is said that approximately 40 per cent of the strains of colon bacilli split sucrose. The recognition and significance of the non-sucrose fermenting strains presents no difficulty; those strains which utilize sucrose, however, present the constant danger of confusion with the aerogenes types which are also frequently present in water supplies.

The colon bacillus is a commensal inhabiting the intestinal

[1] An abstract of this paper was originally presented at the Ithaca Meeting of the American Chemical Society, September 1924.

tract of man and other vertebrates and except under very unusual conditions is incapable of multiplication as a free living saprophyte,—therefore its presence elsewhere may be assumed to be indicative of fecal pollution of human or animal origin.

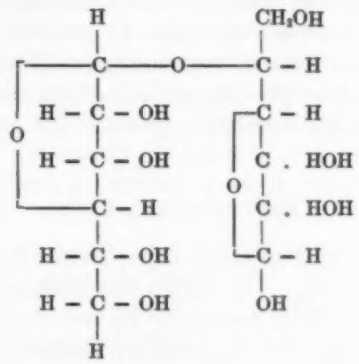
The aerogenes bacillus on the other hand, while also frequently present in the intestine is a free living saprophyte widely distributed in nature. Its presence, therefore, in water is not indicative of fecal pollution. Hence the differentiation of sucrose fermenting strains of colon bacilli from strains of the aerogenes type is both of academic interest and of practical importance.

At present the grouping of a strain may be based on several physiological tests, no one of which is of itself entirely conclusive. The formation of acetyl-methyl-carbinol as determined by the Voges-Proskauer (1898) test, and the ability to grow in Koser's (1918) uric-acid medium, are criteria commonly used. Another criterion, usually termed the "methyl red test" (Clark and Lubs, 1915) depends on the differences in H-ion concentration which are caused by the action of *E. coli* and *A. aerogenes* on *d-glucose* broth. Very recently B. H. Butcher (1924) of Iowa has proposed the use of chinic acid and Koser of Illinois has suggested the use of citric acid media for the differentiation. Of these tests, the V.-P. test for acetyl-methyl-carbinol enjoys at present the official sanction of the committee of the S. A. B. and the generic classification into the *Genera Escherichia* and *Aerobacter* is based on the results of this test.

Chen and Rettger (1920) and others (see bibliographies of Chen and Rettger 1920 and Rogers), have shown that all of these tests are misleading in certain cases and this fact is so generally recognized that critical differentiation is always based on correlated evidence.

The present paper deals with a test which from studies so far completed appears to differentiate sharply between *E. coli* and *A. aerogenes*. It depends upon the use of a sugar, *cellobiose*, which was first obtained from cellulose by Skraup and Koenig, (1901) and which has proved of marked interest to investigators of the constitution of cellulose. The researches of Haworth and Hirst (1921) and of Bergmann and Schotte (1921) have shown

clearly that cellobiose is a glucosido—5-glucose having the following structure:



The fact that cellobiose is hydrolyzed by emulsin indicates that the sugar is a β-glucoside.

While cellobiose is ordinarily obtained in the form of its octaacetate in the acetolysis of cellulose, it has also been reported by Pringsheim (1923) among the hydrolytic products obtained from cellulose by the action of various microörganisms including the denitrifying, methane and hydrogen producing bacteria, as well as the thermophylic cellulose bacteria and cellulose destroying fungi.

Evidently all of these organisms elaborate a *cellulase* which functions over a wider range of temperature than does the accompanying *cellobiase* (which hydrolyzes cellobiose into two molecules of *d*-glucose). In the case of thermophylic bacteria, Pringsheim carried out his hydrolytic fermentation of cellulose at 67°, and was able to arrest the degradation of cellulose at the cellobiose stage without causing it to be hydrolyzed further to glucose.

Despite the great theoretical interest attached to cellobiose we have found no evidence that it has been utilized in practical bacteriological work.

We have found as a new and interesting property of cellobiose that the sugar is rapidly utilized by *A. aerogenes* with acid and gas

formation and that no such changes are brought about by any strains of *E. coli* examined by us. On the basis of these differences in behavior our test for distinguishing between *E. coli* and *A. aerogenes* has been devised. It involves no new technic, is very simple, and (for the strains examined) absolutely conclusive. The method for the preparation of cellobiose, and the procedure and tabulated results are given in the experimental part of this paper.

EXPERIMENTAL PART

The cellobiose[2] used in these experiments was prepared from cellobiose octaacetate. The latter was obtained from cellulose (cotton cellulose or filter paper) by an acetolysis that was practically identical with that described by Madsen (1917) and which has been given in detail by Wise and Russell (1922).

The conversion of the octaacetate was carried out by gradually stirring 20 grams of the cellobiose octaacetate into 150 cc. of a cold 10 per cent solution of potassium hydroxide in 95 per cent alcohol. The white crystalline monopotassium derivative was filtered, washed with absolute alcohol and dissolved in ice water. This alkaline solution was then immediately titrated to the neutral point with dilute acetic acid, filtered and concentrated nearly to dryness in *vacuo*. The residue was then crystallized from ethyl alcohol or methanol and was usually found to be sufficiently pure for our purposes. It could be further purified by solution in water and precipitating by the addition of acetone.

Needless to say, all precautions must be taken to avoid hydrolysis of the disaccharide to glucose but in general these conditions are easily met. Resinification of the sugar (especially in alkaline solution) must also be avoided. The cellobiose used in our experiments was a colorless or faintly tinted microcrystalline powder, melting at 225°C. (uncorrected).

[2] The cellobiose used in our experimental work was prepared by Mr. Floyd Peterson, graduate assistant in Forest Chemistry at the New York State College of Forestry. Our thanks are due to Mr Peterson for his enthusiastic coöperation throughout the course of this work. His results on the preparation of cellobiose will be published elsewhere.

The cellobiose medium used was prepared by the addition of 0.5 per cent of the sugar to nutrient broth containing 0.3 per cent Difco beef extract and 1 per cent Difco pepton. Sterilization was carried out by autoclaving for 20 minutes at approximately 121°C. after distribution in fermentation tubes of either the Smith or the Dunham type. Results were observed after twelve, twenty-four, forty-eight and one hundred and twenty hours incubation at 37°. Obviously the sugar may be added to any sort of liquid or liquefiable media just as other sugars are, and in the same proportions. There is such an abundant gas production, however, in a broth containing 0.5 per cent that there would seem to be no advantage in using more and probably a much smaller proportion would be equally satisfactory.

Tables 1 and 2 indicate the results obtained with known strains of *E. coli* and *A. aerogenes*. It will be noted that in every case the strains of *A. aerogenes* have split the cellobiose with gas and acid production, while neither acid nor gas was produced by *E. coli*.

It will be noted that with the 52 organisms in the series, the Voges-Proskauer test correlates with the cellobiose test in every case with the exception of *E. neopolitana* 602 (Series I). The ability of this strain of *E. neopolitana* to utilize cellobiose may be interpreted as circumstantial evidence that the strain is an aberrant species of the genus Aerobacter.

It will be noted also that the organism designated as *A. aerogenes* S. gives a positive methyl-red test with a positive Voges-Proskauer and a positive reaction with cellobiose.

The results obtained in these studies seem to indicate that the use of cellobiose affords a means of differentiating the genera Escherichia and Aerobacter that is fully equal to those now in use in dependability and superior to them in simplicity and in the ease of interpretation.[3]

[3] The cultures used in Series I were supplied by Dr. L. F. Rettger of Yale University and Dr. M. Levine of the University of Iowa, to whom our thanks are due.

TABLE 1

Series 1. Reactions of a series of stock strains of the colon-aerogenes group on cellobiose

ORGANISM	SOURCES	VOGES-PROSKAUER TEST	METHYL-RED TEST	CELLOBIOSE
E. coli 434	Human feces	Negative	Positive	Negative
E. coli 436	Human feces	Negative	Positive	Negative
E. coli 442	Human feces	Negative	Positive	Negative
E. coli 446	Human feces	Negative	Positive	Negative
E. coli 460	Human feces	Negative	Positive	Negative
E. coli 465	Human feces	Negative	Positive	Negative
E. coli S	Sheep feces	Negative	Positive	Negative
E. coli C	Cow feces	Negative	Positive	Negative
E. coli H	Horse feces	Negative	Positive	Negative
E. coli CH	Chicken feces	Negative	Positive	Negative
E. coli M	Monkey feces	Negative	Positive	Negative
A. cloacae		Positive	Negative	Acid and gas (25 per cent)
A. aerog.	Human feces	Positive	Negative	Acid and gas (50 per cent)
A. aerog.	Rat feces	Positive	Negative	Acid and gas (50 per cent)
A. aerog. 1	Soil	Positive	Negative	Acid and gas (35 per cent)
A. aerog. 3	Soil	Positive	Negative	Acid and gas (40 per cent)
A. aerog. S	Soil	Positive	Positive	Acid and gas (50 per cent)
E. coli La		Negative	Positive	Negative
E. coli 24		Negative	Positive	Negative
E. paragrunthali 36		Negative	Positive	Negative
E. communior 41		Negative	Positive	Negative
E. coscoroba 70		Negative	Positive	Negative
E. acidi-lac. 43		Negative	Positive	Negative
E. acidi-lac. 165		Negative	Positive	Negative
E. neapolitani 594		Negative	Positive	Negative
E. neapolitani 602		Negative	Positive	Acid and gas (50 per cent)
A. cloacae 233	Soil	Positive	Negative	Acid and gas (95 per cent)
A. cloacae 235	Human feces	Positive	Negative	Acid and gas (95 per cent)
A. aerog. 252	Soil	Positive	Negative	Acid and gas (75 per cent)
A. aerog. 80	Soil	Positive	Negative	Acid and gas (50 per cent)

TABLE 2

Series 2. Reactions of a series of colon-aerogenes strains on cellobiose

ORGANISM	SOURCE	VOGES-PROSKAUER TEST	METHYL-RED TEST	SUCROSE	CELLOBIOSE
E. coli 1	Human feces	Negative	Positive	Negative	Negative
E. coli 2	Human feces	Negative	Positive	Negative	Negative
E. coli 3	Human feces	Negative	Positive	Negative	Negative
E. coli 4	Human feces	Negative	Positive	Negative	Negative
E. coli 5	Human feces	Negative	Positive	Negative	Negative
E. coli 6	Human feces	Negative	Positive	Acid and gas	Negative
E. coli 7	Human feces	Negative	Positive	Acid and gas	Negative
E. coli 8	Human feces	Negative	Positive	Negative	Negative
E. coli 9	Human feces	Negative	Positive	Negative	Negative
E. coli 10	Human feces	Negative	Positive	Negative	Negative
E. coli 11	Horse feces	Negative	Positive	Negative	Negative
E. coli 12	Horse feces	Negative	Positive	Negative	Negative
E. coli 13	Horse feces	Negative	Positive	Negative	Negative
E. coli 14	Horse feces	Negative	Positive	Negative	Negative
E. coli 15	Horse feces	Negative	Positive	Acid and gas	Negative
E. coli 16	Water	Negative	Positive	Acid and gas	Negative
E. coli 17	Water	Negative	Positive	Acid and gas	Negative
A. aerog. 18	Human feces	Positive	Negative	Acid and gas	Acid and gas (50 per cent)
A. aerog. 19	Water	Positive	Negative	Acid and gas	Acid and gas (75 per cent)
A. aerog. 20	Water	Positive	Negative	Acid and gas	Acid and gas (30 per cent)
A. aerog. 21	Water	Positive	Negative	Acid and gas	Acid and gas (50 per cent)
A. aerog. 22	Horse feces	Positive	Negative	Acid and gas	Acid and gas (50 per cent)

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THE ELECTROPHORETIC MIGRATION OF VARIOUS TYPES OF VEGETABLE CELLS[1]

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PREVIOUS KNOWLEDGE IN REGARD TO THE ELECTROPHORETIC MIGRATION OF VEGETABLE CELLS

During the latter part of the nineteenth century a number of more or less elementary observations were made in regard to the behavior of protozoa under the influence of electrical currents—work which is summarized by Dale (1901). Following the studies of Hardy (1899; 1900) on the influence of electrical charge upon the coagulation of colloidal particles, more active progress was made in the study of this problem and the work was extended to cover other unicellular organisms. Fischer (1900) described the effect of weak currents upon the infusoria and predicted that bacteria would behave in a similar fashion, although, because of their minute size, experimentation in this case would be more difficult. Bechhold (1904) was apparently the first, actually to observe and record the fact that bacterial cells do carry a negative charge. Lillie (1903) determined the direction of migration of various isolated cells in the path of a current and stated that cells with abundant nuclear material migrated to the anode, as did silica dust, while cells with voluminous cytoplasm, such as certain red blood cells, leucocytes and muscle cells travelled with the positive stream. The latter result has not been confirmed by later workers. Greeley (1904) reported exhaustive studies in regard to the migration of protozoa which brought out the influence of acidity and alkalinity upon the direction of movement.

[1] Thesis presented by the junior author in candidacy for the degree of Doctor of Philosophy at Yale University.

Neisser and Friedemann (1904) observed the negative charge on bacterial cells. Teague and Buxton (1906) discovered that an increase in hydrogen ion concentration causes a decrease in charge and that with continuing increase in acidity the charge is ultimately reversed so as to become positive. (See also Buxton and Shaffer, 1906.) Cernovodeanu and Henri (1906) studied a larger series of bacteria than any of their predecessors and reported that all of the species except *B. dysenteriae* (Flexner), as well as a mold, carried a negative charge. The claim that the dysentery bacillus is electro-positive has since been contradicted by Szent-Györgyi (1921). The forms of apparatus used by many of these earlier investigators (generally involving macroscopic observation of the aggregation of bacteria at one or the other end of a U-tube) did not lend themselves to exact quantitative results. A glass cell adapted to more accurate microscopic studies was introduced by Lortet (1896) used by Russ (1909) and Thornton (1910) and perfected by Powis (1914) and by Northrop (1922). Hardy and Harvey (1911) first employed non-polarizable electrodes in work of this kind.

Many important studies on the electrophoretic migration of bacteria have been conducted during the past few years with the use of the technique thus devised. A fairly extensive bibliography has been presented elsewhere by Winslow, Falk and Caulfield (1923); and it seems unnecessary to review the literature again in detail. Three communications of some significance have, however, appeared since the paper to which reference has just been made (Eggerth, 1923, 1924; Winslow and Shaughnessy, 1924; Winslow and Fleeson, 1925).

The net result of the work which has been done to date may be briefly summed up as follows:

A. Unicellular organisms (whether living or dead), as well as certain inorganic particles, bear a negative charge. Abbott (1908) and Thornton (1910), Cernovodeanu and Henri (1906)—in the case of *B. dysenteriae* only, and Szent-Györgyi (1921)—in the case of a fungus from the stomach of a mouse—report positive charges but the evidence on the other side is, in general, so strong as to make it likely that these observers were in error.

It is quite probable that they may have been misled by the phenomenon of electrical endosmose in the vicinity of the glass.

B. The negative charge on the cell is increased by very slight additions of certain neutral salts (Winslow and Fleeson, 1925).

C. The charge on the cells is markedly decreased with higher concentrations of salts or with the addition of acid or alkali (Northrop and DeKruif, 1922; Winslow, Falk and Caulfield, 1923; Winslow and Fleeson, 1925).

D. The charge is reversed and becomes positive with very high concentration of acids or alkalies or with some bivalent and trivalent salts (Northrop and DeKruif, 1922; Winslow and Shaughnessy, 1924; Winslow and Fleeson, 1925).

E. The effects noted under (C) are solely, or at least predominantly, due to the cation (Winslow and Fleeson, 1925).

F. In general, the effect of the cation increases with its valency but hydrogen is more active than other monovalent ions (Northrop and DeKruif, 1922; Winslow and Fleeson, 1925).

G. The presence of a monovalent ion tends to neutralize the effect of a bivalent ion and the presence of a positive ion other than hydrogen tends to some extent to neutralize the influence of the hydrogen ion itself (Winslow, Falk and Caulfield, 1923).

OBJECTIVES OF THE PRESENT INVESTIGATION AND TYPES OF ORGANISMS STUDIED

The object of the present study was to discover the extent to which the reactions noted above could be discovered to be specific for various types of vegetable cells. Certain previous workers had observed one or two organisms with care and others had recorded merely qualitative results, or results at certain limited reaction ranges for a larger series of forms. It was our purpose to select a dozen or more vegetable microörganisms, representing widely separated biological types, and to study their electrophoretic migration under a wide range of pH values in order to determine how far in the words of Loeb (1922) the phenomena of cataphoretic charge are due to "forces inherent in

the water itself" and how far they are affected by the specific physical and chemical properties of the suspended cell.

In the conduct of these studies we used three fundamental menstrua, distilled water, Ringer's solution and a solution of 0.145 M NaCl (isotonic with Ringer's solution), observations being made in each of the three menstrua at reactions between pH 1.5 and pH 12.5.

Complete tests were made in this fashion with twelve different organisms and with silica dust particles; and in addition, the results obtained with our primary strain of streptococcus were checked by observations at a few typical pH values of four additional strains of streptococci, obtained from diverse sources and possessing diverse biological attributes.

The thirteen primary series of experiments were conducted with the following types of organisms (or inorganic suspended particles):

1. Particles of silica dust. Finely divided particles of silica dust from material used by Dr. Leonard Greenburg in electrophoretic studies. They ranged from 6 to 10 microns in size.

2. Chlorella. A one-celled green alga obtained from Dr. F. B. Wann of the New York State College of Agriculture, Cornell University, labelled no. 11. This organism grew readily on one per cent glucose agar in sunlight at room temperature. Its cells were about 6 microns in diameter.

3. Saccharomyces apiculatus. A typical small yeast obtained from the Department of Botany of the Michigan Agricultural College. It grew readily on glucose agar at room temperature. Its cells were about the same size as those of Chlorella and as the particles of the silica dust. This is the same strain studied by Winslow and Fleeson (1925).

4. Streptococcus pyogenes. A long-chained non-hemolytic organism, originally isolated from pus, courteously provided by Professor L. F. Rettger of the Department of Bacteriology of the Yale Graduate School.

5. Salt-tolerant organism. Salt-tolerant organism no. 4 obtained from Dr. W. S. Sturges of the Cudahy Packing Company laboratories. This organism (a large rod about 2 microns long

by 1 micron wide) is characterized by its power to grow in the presence of 2 M NaCl; but it was easily cultivated on either plain or glucose agar at 37°C. It was thought that the power of this organism to resist the toxic effect of salt would make its reactions of interest.

6. Clostridium flabelliferum. This is the "fish-tail" anaerobe described by Sturges (1926) and was obtained from his laboratory at the Cudahy Packing Company. It was selected, after considerable experimentation with other species, as a representative of the group of obligate anaerobes which, although a spore former, forms spores so slowly that it was easy to obtain cultures containing only vegetative cells. It is a large rod and was cultivated by anaerobic methods (in presence of pyrogallic acid and caustic potash on glucose agar at 37°C.)

7. Mycobacterium smegmatis. This organism was used as a type of the acid-fast group. It was obtained from the laboratories of the New York State Department of Health, Albany, N. Y. Satisfactory growth was made on one per cent glucose agar at 37° in from four to ten days.

8. Bacillus cereus. The same strain of aerobic spore former used in previous studies from this laboratory (Winslow, Falk and Caulfield, 1923; Winslow and Shaughnessy, 1924.) Five-hour cultures at 37°, or eighteen-hour cultures at room temperature, on plain nutrient agar, were found to be almost entirely composed of vegetative cells.

9. Pseudomonas pyocyanea. Obtained from Prof. G. H. Smith of the Department of Pathology and Bacteriology of the Yale Medical School. This organism was of course Gram-negative and had the smallest cells of any type studied, a fact which made it very hard to observe. It was grown on plain nutrient agar for twenty-four hours at 37°C.

10. Bacterium coli I. A strain isolated by Cohen in 1922 and used in previous work from this laboratory (Winslow and Shaughnessy, 1924; Winslow and Fleeson, 1925). These organisms were close to the limit of vision and appeared to grow smaller as the experiments proceeded and results are therefore less accurate than those obtained with larger forms. The organism was grown on plain nutrient agar for twenty to thirty hours at 37°C.

11. Bact. coli II. A second strain, isolated from sewage in this laboratory in 1924.

12. Lactobacillus acidophilus. Several different strains of this organism were used, but, as no significant differences were observed, the results have been grouped together. All were obtained from Prof. L. F. Rettger of the Department of Bacteriology in the Yale Graduate School. They were cultivated for forty-eight hours at 37°C. on one per cent galactose tryptic-digest agar. In preparing this medium 100 cc. "Klim" in 1000 cc. of 1 per cent sodium carbonate solution was allowed to digest with Fairchild's Trypsin powder for two days at 37°C. After adjusting to pH 6.5, 100 cc. of the digest were added to 1 liter of water containing 5 grams of beef extract. Stock cultures were maintained in milk.

13. Capsulated streptococcus. A short chained non-hemolytic Gram-positive bile-insoluble streptococci with well-developed capsules. It was a stock culture courteously furnished by Prof. F. P. Gorham of Brown University. This organism agglutinates spontaneously in solutions on the acid side of the neutral point.

TECHNIQUE OF THE EXPERIMENTS

The organisms to be used in an experiment were in all cases cultivated on a solid agar medium in Kolle flasks or large bottles—the constituents of the agar and the time and temperature of incubation for each species being as indicated in preceding paragraphs. After the proper period of incubation, the cells were washed off in distilled water (or in Ringer's solution or 0.145 M NaCl as the case might be) and then centrifuged and re-suspended in the appropriate menstruum three times. Before making the final suspension the organisms were shaken up with glass beads in a small amount of menstruum and filtered through cotton to remove large clumps or flakes of agar. The final suspension was of such a density as to appear slightly cloudy to the naked eye and to give a few organisms in each microscopic field.

Readings of migration velocity were first made at the initial pH of the suspension, which was about pH 5.4 in water and NaCl

solutions, and about pH 8.0 in Ringer's solution. Adjustment was then made to the various other pH values desired by addition of HCl or NaOH. The pH thus obtained was of course more or less unstable, particularly near the neutral point. The actual pH of the suspension leaving the electrophoretic cell was therefore determined and this value taken as corresponding to the recorded velocities. Readings of pH were made by comparison with the color plates of Clark (1922) while above pH 9.8 the alkaline indicators of Prideaux (1917) were used; although the buffer standards for these indicators proved highly unsatisfactory, on account of their rapid deterioration.

The apparatus used for all determinations was the microscopic cell devised by Northrop (1922) and used with slight modifications by Winslow and Fleeson (1925). A detailed description may be found in the reports of these investigators and need not be repeated here. Suffice it to say that the suspension was run through a glass chamber 1 cm. wide, 7 cm. long and 1 mm. deep, connected at each end with zinc-zinc-sulphate electrodes. Every precaution was taken to secure the highest degree of cleanliness in the apparatus and the cell was freed from air bubbles and thoroughly flushed with each particular menstruum studied before a set of observations was made. A direct current of 112 volts and 12 milliamperes was applied through the non-polarizable electrodes, giving a fall in potential within the cell of about 13 volts per centimeter.

Observations were made with direct illumination and a magnification of 525 diameters. Two different ocular micrometers were used, bearing rulings respectively 30 and 44 micra apart. The rate of migration of five different cells was recorded by the use of a stop-watch at each level observed and the average of the five readings taken as the velocity at that level. The apparatus was always carefully levelled and any possible error due to drifting detected by frequent reversal of the current. In the highly alkaline solutions this was an important precaution since leakage not infrequently occurred as a result of the action of the alkali on the DeKhotinsky cement with which the parts of the cell were joined.

A most important problem to be considered in work of this kind is the level at which observations are to be made. As a result of the phenomena of electrical endosmose, the potential difference between water and glass causes a streaming of the water toward the cathode near the upper and lower glass surfaces of the cell, balanced by a streaming toward the anode in its middle depths. Thus the true velocity of migration of negatively-charged particles will be accelerated at mid-depth and retarded or reversed in the upper and lower levels of the cell. In previous work it has been usual to average readings taken at the mid-points of the three lower sixths or eighths of the cell. For reasons discussed by Winslow and Fleeson (1925) we have concluded that it is more accurate to average readings taken at the mid-points of the upper and lower halves of the cell. The value recorded for any given menstruum in any given experiment was therefore based on the migration velocity of at least 10 different particles, 5 in the upper half and 5 in the lower half of the cell. As a rule four or five experiments were conducted with each organism in each different menstruum, so that the average values given in the tables represent 40 or 50 recorded velocities. In the few cases where only one experiment was made at a given pH the value in the tables is enclosed in brackets and these values have been omitted from the charts.

Throughout this work results have been expressed in terms of velocity in micra per second since as Winslow, Falk and Caulfield (1923) have pointed out there is some possibility of error in the assumption of the constants of the Helmholtz-Lamb equation, by which such velocities are converted into terms of charge upon the migrating particles. Curiously enough, however, the dimensions of the cells and the current-strength used were such that, if the usual assumptions as to dielectric constant and other factors be made (see Winslow, Falk and Caulfield, 1923) the observed migration velocities correspond directly and quantitatively to millivolts of potential difference between suspended particles and menstruum.

PRESENTATION OF DETAILED RESULTS

The average migration velocities observed at each pH value in each of the three menstrua studied are presented below in table 1 (water), table 2 (Ringer's solution) and table 3 (0.145 M NaCl); and the results are presented graphically in figures 1 to 3. In all charts, ordinates represent velocities in micra per second, and abscissae represent pH values.

GENERAL CHARACTERISTICS OF ELECTROPHORETIC MIGRATION AS INDICATED BY OUR RESULTS

It will be evident from a survey of tables 1 to 3 and figures 1 to 3 that our results, so far as the general phenomena of electrophoretic migration are concerned, are in complete harmony with those of previous workers in this field. We find that all of the suspended particles studied, whether organic or inorganic, living or dead (the microörganisms must of course have been killed by the more extreme acidities and alkalinities employed) bear a negative charge at ranges of hydrogen ion concentration above pH 2.0.

This charge reaches a maximum at two points on either side of neutrality. The exact relation of the two maxima, and the slight dip between them, varies somewhat, as would be expected, since the reaction of an unbuffered solution near the neutral point is so difficult to adjust with accuracy, *B. cereus* and *Bact. coli* I show a smooth curve with a maximum at neutrality while *St. pyogenes* shows a peak on the alkaline side and *Bact. coli* II a peak on the acid side of neutrality. For *Ps. pyocyanea* there are too few data to make our results significant. In all other cases (*L. acidophilus*, *Cl. flabelliferum*, salt-tolerant organism, *Sacch. apiculatus*, *Chlorella*, *Myco. smegmatis*, the capsulated streptococcus and silica dust) we find a fairly high velocity at neutrality which rises to a maximum on either side of the neutral point. The same phenomena are shown by the data of Winslow, Falk and Caulfield (1923) and Winslow and Fleeson (1925). We believe it to be a general one and are inclined to assume that

TABLE 1

Average migration velocities (in micra per second) of various vegetable microörganisms and silica dust particles at various pH values

Suspended in distilled water

	pH											
	1.5	2.5	3.5	4.5	5.5	6.5	7.5	8.5	9.5	10.5	11.5	12.5
Silica dust	−10.8	−14.7	−16.2	−15.8	−13.8	−14.0	−15.0	−21.5	−22.1			−19.1
Chlorella	−1.5	−4.0	−13.8	−14.4	−19.8	−23.1	−17.5	−21.6	−18.1			−13.5
Sacch. apiculatus	−4.1	−5.4	−7.6	−7.7	−20.3	−19.9	−17.1	−15.0	−17.2		−10.0	−5.9
St. pyogenes	−0.9	−2.9	−9.4	−14.1	−17.2	−17.7	−18.7	−24.5	−20.4	−20.0	−15.8	−9.7
Salt-tolerant organism	−0.3	−0.2	−11.1	−13.4	−15.5	(−22.1)	−17.2	(−19.3)	−18.6			−9.9
Cl. flabelliferum	+1.1	+1.1	−9.5	−9.8	−19.6		−15.1	(−17.9)	−23.2			−17.6
Myco. smegmatis	+3.0	+4.3	−3.5	−13.0	−23.5	−19.0	−20.4	−26.3	−22.4	−22.2	−14.9	−9.3
B. cereus	+1.7	+4.7	−1.0	−12.9	−14.0	−15.4	−19.6	−18.3	−18.5		(−10.3)	−11.2
Ps. pyocyanea	(+2.9)	(+1.9)	(−13.0)		(−15.0)	(−15.0)		(−27.0)				(−10.0)
Bact. coli I	0	−1.9	−6.0	−12.4	−11.8	−14.5	−14.9	−14.1	−11.9	(−5.1)	−7.3	−5.1
Bact. coli II	+1.4	0	−9.5	−8.4	−13.2	(−20.0)	−15.0	(−9.4)	−10.8			−8.9
L. acidophilus	+2.3	+2.4	−4.0	−12.1	−14.6	−17.2	−13.4	−18.8	−14.8	−17.0	−15.0	−10.3
Capsulated streptococcus	+2.1	+0.1	−1.4	−11.3	−6.1	−8.2	−9.7	−12.9	−9.3			−9.5

+ indicates migration toward cathode; − indicates migration toward anode.
Figures in parentheses based on a single experiment only and omitted from the charts.

TABLE 2

Average migration velocities (in micra per second) of various vegetable microörganisms and silica dust particles at various pH values

Suspended in Ringer's solution

	pH											
	1.5	2.5	3.5	4.5	5.5	6.5	7.5	8.5	9.5	10.5	11.5	12.5
Silica dust	−9.1	−10.0	−10.4	−14.0	−14.6	−17.1	−16.5	−14.7	−13.4			−10.9
Chlorella	−7.5	−9.7	−12.3	−10.7	−10.4	−12.7	−12.5	−13.0	−12.3			−12.7
Sacch. apiculatus	−5.8	−5.8	−6.8	−7.8	−8.2	−7.5	(−7.4)	−9.2	−8.4		−9.4	−8.2
St. pyogenes	−5.7	−6.2	−7.2	−8.6	−9.2	−9.7	−9.8	−10.6	−11.2	−10.7	−10.0	−9.9
Salt-tolerant organism	−6.6	−7.2	−10.4	(−9.3)	−12.0	−13.6	−12.4	−14.1	(−14.4)			−13.2
Cl. flabelliferum	−0.8	−1.7	−3.7	(−9.7)	−9.3	−10.5	−11.5	−11.3	−13.8			−12.0
Myco. smegmatis	+1.4	+1.4	−2.9	−4.0	−11.3	−10.3	−12.3	−9.9	−14.5			−10.0
B. cereus	−5.7	−6.2	−6.8	−7.7	−8.6	−9.5	−7.8	(−11.1)	−8.5			−9.7
Ps. pyocyanea	0	0	−4.5	−6.2		−8.6		−7.1	−7.1			−8.7
Bact. coli I	−0.8	−1.0	−2.2	−3.8	−3.2	−4.9	−5.7	−5.2	−4.9		−7.9	−5.1
Bact. coli II	(−2.8)		(−4.3)	−4.7	(−5.9)	(−6.6)		−5.7				(−6.8)
L. acidophilus	+0.6	+0.1	−0.9	−4.3	−4.5	−5.8	−5.4	−7.2	−8.5	−6.9		−7.4
Capsulated streptococcus	−1.5	−3.7	0.0	−2.4	−6.2	−3.5	−6.1	−4.8	−6.1			−5.9

+ indicates migration toward cathode; − indicates migration toward anode.

Figures in parentheses based on a single experiment only and omitted from the charts.

TABLE 3

Average migration velocities (in micra per second) of various vegetable microörganisms and silica dust particles at various pH values

Suspended in 0.145 M NaCl

	pH											
	1.5	2.5	3.5	4.5	5.5	6.5	7.5	8.5	9.5	10.5	11.5	12.5
Silica dust	−10.1	−12.6	−14.5	−13.9	−14.3	−17.0	−20.1	−16.0	−18.0			−16.4
Chlorella	−7.2	−6.2	−7.7	(−8.3)	−11.4	−11.8	−12.3	−10.4	−11.3			−12.5
Sacch. apiculatus	−4.9	−4.9	−5.6	−7.1	−8.0	−6.3	(−12.5)	−7.8	−8.3			−9.1
Salt-tolerant organism	−5.0	−7.3	−9.5		−14.1	−13.1	(−14.6)	−15.0				−13.0
Myco. smegmatis	−3.9	−4.0	−7.6	(−12.1)	−12.1	(−12.7)	−16.2	(−10.7)	(−15.0)			−12.8
B. cereus	−7.2	−7.8	−9.9	−10.3	−10.8	−12.0	−12.4	−12.4	(−11.3)			−10.4

+ indicates migration toward cathode; − indicates migration toward anode.
Figures in parentheses based on a single experiment only and omitted from the charts.

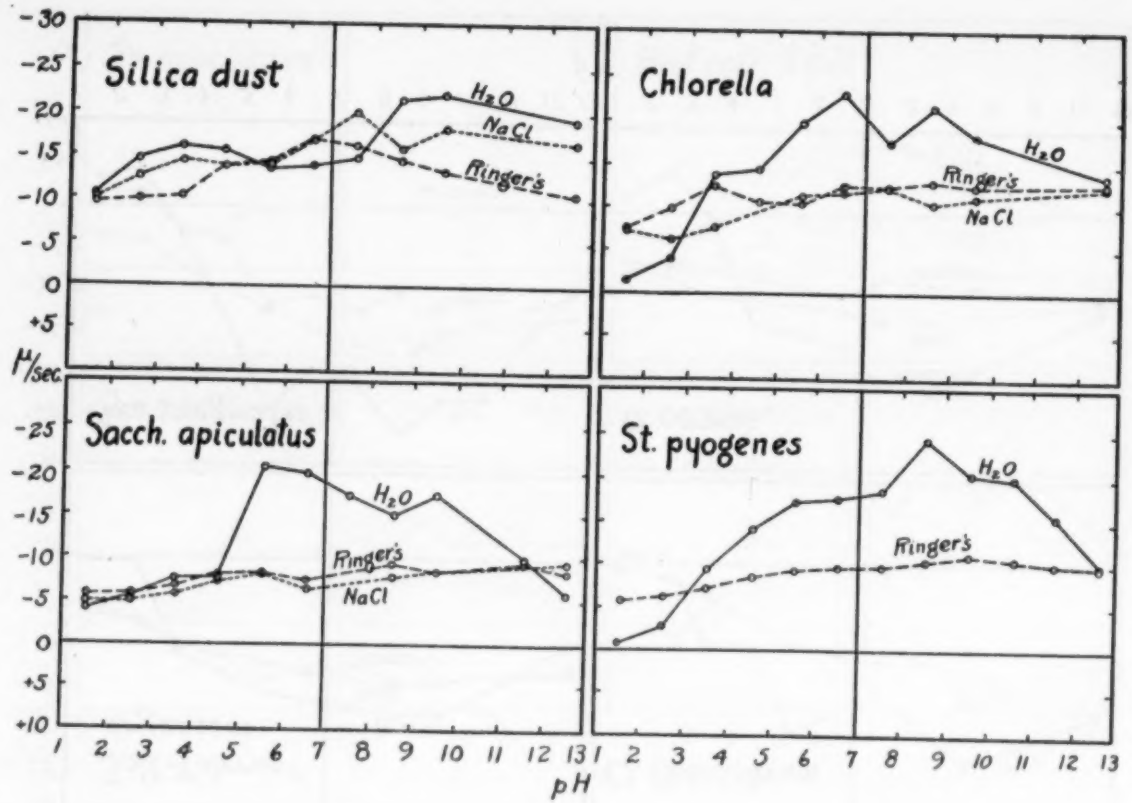
Silica dust
Chlorella
Sacch. apiculatus
St. pyogenes
H₂O
NaCl
Ringer's
μ/sec.
pH
-30
-25
-20
-15
-10
-5
0
+5
+10
1 2 3 4 5 6 7 8 9 10 11 12 13

FIG. 1

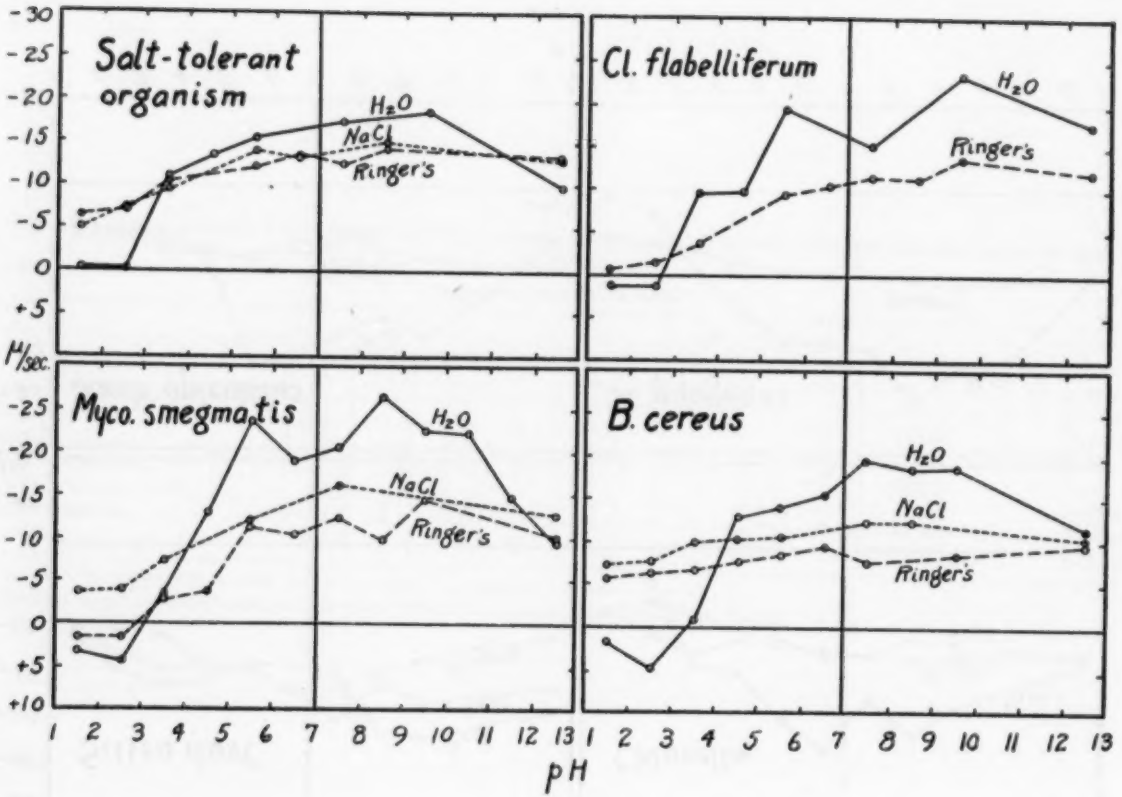
Salt-tolerant organism
Cl. flabelliferum
Myco. smegmatis
B. cereus
H₂O
NaCl
Ringer's
μ/sec.
pH
-30
-25
-20
-15
-10
-5
0
+5
+10
1 2 3 4 5 6 7 8 9 10 11 12 13

FIG. 2

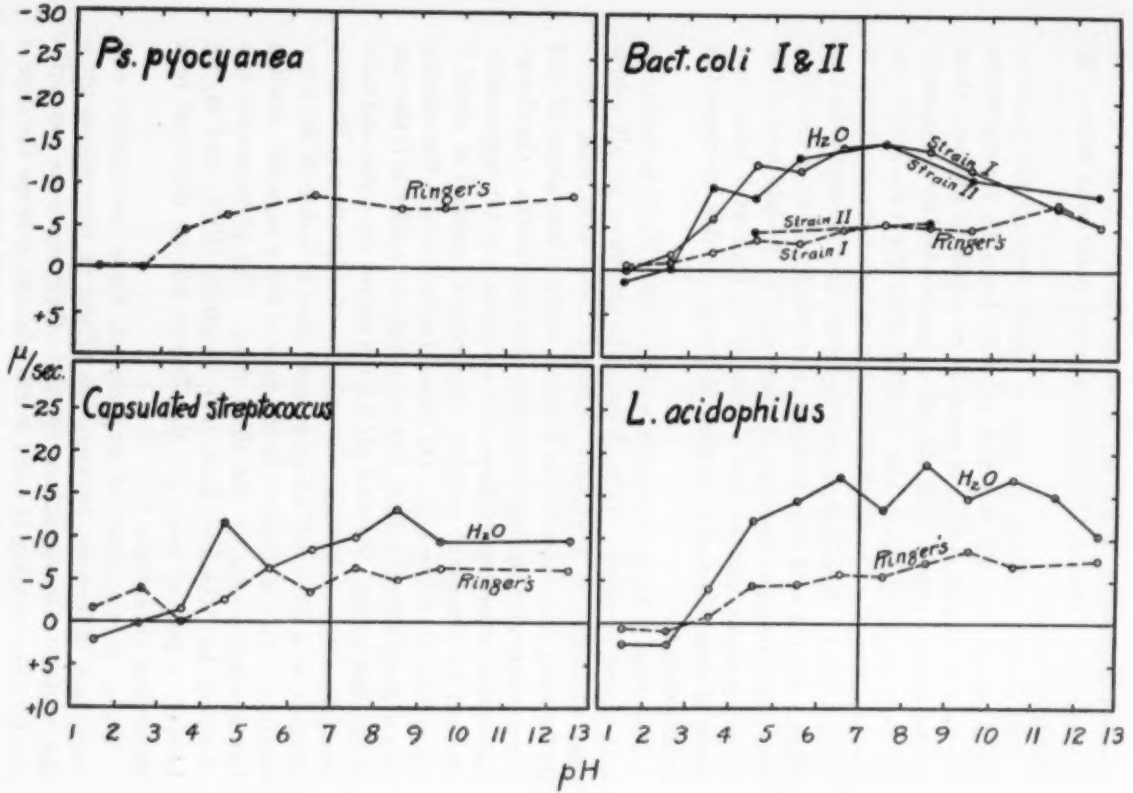
Ps. pyocyanea
Ringer's
Bact. coli I & II
H₂O
Strain I
Strain II
Strain II
Strain I
Ringer's
Capsulated streptococcus
H₂O
Ringer's
L. acidophilus
H₂O
Ringer's
μ/sec.
-30
-25
-20
-15
-10
-5
0
+5
+10
1 2 3 4 5 6 7 8 9 10 11 12 13
pH

FIG. 3

where a depression in the curve at the neutral point is not recorded, it is because the actual neutral point was missed in making the tests.

The further addition of acid or alkali (beyond the maxima which occur near pH 6.5 and pH 8.5) leads to a progressive decrease in charge, which is much more rapid on the acid than on the alkaline side. With the pH values studied by us (extending only to pH 12.5 on the alkaline side) the velocity of the particles decreased in alkaline solution only to between 5 and 14 micra while *Cl. flabelliferum* and the silica dust showed even less effect. On the acid side, however, the velocity of even the silica dust was reduced to less than 11 micra at pH 1.5 while none of the microörganisms studied had a velocity as high as 5 micra and seven of them showed a reversal of charge, at this hydrogen ion concentration.

The effect of Ringer's solution and of 0.145 M NaCl is similar to that of acid or alkali. In both cases the charge at all pH values near neutrality is markedly reduced. The maximum velocity for silica dust observed in Ringer's solution was about 17 and the maximum for any microörganism was 14.5 micra. On the alkaline side, velocities in these salt solutions are not appreciably affected by change in reaction, remaining in general at about 9 micra from pH 8.5 to 12.5. On the acid side, however, the velocity falls with additional acidity, but not nearly so rapidly as is the case in distilled water. Beyond pH 3.5 the curves for water and salts generally cross each other, the velocity of migration in Ringer's solution at a given pH being greater than in water of the same reaction. This is manifest in the case of every organism studied but was not shown by the silica dust. The phenomenon was observed by Winslow, Falk and Caulfield (1923) and is, we believe, a general one. Its significance will be discussed in a succeeding paragraph.

While, as will later be emphasized, there are distinct and important differences between the effects of electrolytes upon the various microörganisms studied, their reactions are sufficiently alike to make it seem worth while to average the results obtained with different species so as to present a rough composite

TABLE 4

Average velocities of all microörganisms studied in each menstruum at various pH values

		pH											
		1.5	2.5	3.5	4.5	5.5	6.5	7.5	8.5	9.5	10.5	11.5	12.5
5 organisms*	0.145 M NaCl	−5.6	−6.0	−7.9	−9.7	−11.3	−11.1	−13.6	−11.3	−11.5			−11.6
	Ringer's	−4.8	−5.5	−7.8	−7.9	−10.1	−10.7	−10.5	−11.5	−11.6			−10.8
11 organisms†	Ringer's	−3.1	−3.6	−5.6	−7.0	−8.3	−9.1	−9.4	−9.5	−10.4	−8.8	−9.1	−9.4
	Water	+0.5	0.0	−7.1	−11.8	−16.8	−18.4	−16.9	−19.3	−17.7	−16.1	−10.5	−10.1

* Chlorella, salt-tolerant organism, *Sacch. apiculatus*, *Myco. smegmatis*, *B. cereus*.

† Five organisms listed under (1) plus *St. pyogenes*, *Cl. flabelliferum*, *Ps. pyocyanea*, *Bact. coli* I, *Bact. coli* II and *L. acidophilus*.

picture of the group as a whole. This has been done in table 4, comparing in the first two lines the values obtained in NaCl and Ringer's solution for the five species studied in the former menstruum and in the last two lines the values obtained in Ringer's solution and in water for all the organisms studied except the capsule-bearing form. This capsulated organism and the silica dust are so different from the rest in their behavior that they have been omitted from the calculation.

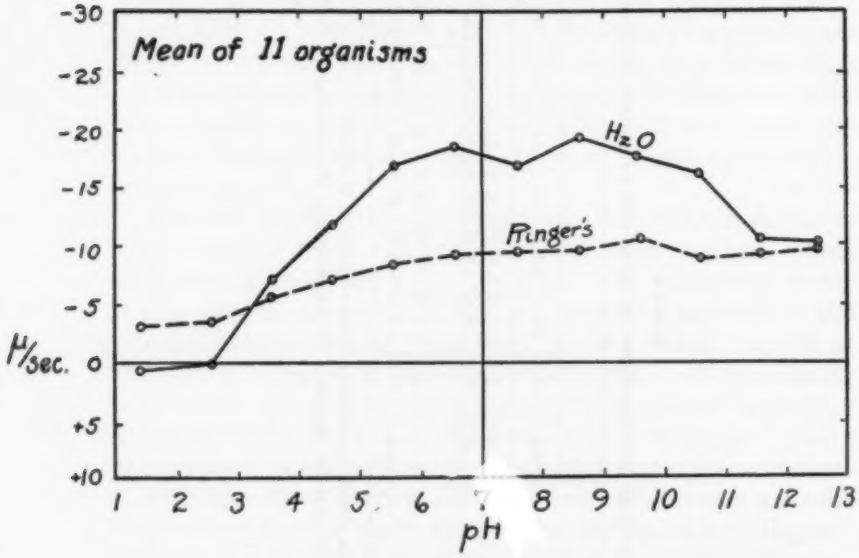

FIG. 4

The results of this composite average, as presented in figure 4, give a very clear picture of the general phenomena observed. We note a migration velocity of about 17 at pH 7.5 rising to 18 at pH 6.5 and to 19 at pH 8.5. On the alkaline side, the composite curve falls with increasing alkalinity to 10 at pH 12.5. On the acid side it falls more rapidly to an isopotential point at pH 2.5 and the charge becomes positive at pH 1.5. In Ringer's solution the composite curve remains between 8 and 10 at all reactions between pH 7.5 and pH 12.5. On the acid side the

velocity falls slowly with increasing acidity to 3 at pH 1.5 but is higher than that observed in corresponding pH ranges in distilled water beyond pH 3.5.

VARIATIONS IN ELECTROPHORETIC MIGRATION CHARACTERISTIC OF VARIOUS TYPES OF SUSPENDED PARTICLES

We may now turn from the general characteristics of the electrophoretic curve to the specific peculiarities of response exhibited by different types of vegetable microörganisms. Reference to tables 1 to 3 and figures 1 to 3 will make it at once clear that such differences actually exist.

First of all, it is evident that the velocity of migration of silica dust is much less influenced by either acid or alkali or salts than in the case of the organic cells here studied. Its maximum velocity of 22.1 in water is reduced only to 10.8 at pH 1.5 and to 19.1 at pH 12.5.—in Ringer's solution at pH 6.5 only to 17.1. The migration velocity of *Chlorella* is more affected by either acid or salt than is that of silica dust and *St. pyogenes* and *Sacch. apiculatus* come next in order. All these organisms as indicated in figure 5 show curves in Ringer's solution which are fairly flat on the alkaline side and dip gently on the acid side but they lie at slightly different levels.

Myco. smegmatis and *Cl. flabelliferum* show a distinctly different type of curve (fig. 6), at a high level on the alkaline side, but falling off sharply on the acid side. These organisms are characterized by a moderate effect of salt (similar to that shown in the *Chlorella* group) but a very marked effect of acid. *B. cereus* and the salt-tolerant organism are probably to be classed in this group, since their curves in water fall very low in the acid range although for some reason they do not dip steeply at the acid end of the Ringer's curve.

A third group (fig. 7) includes *Ps. pyocyanea*, the two strains of *Bact. coli* and *L. acidophilus* which are very markedly affected by both acid and salts, showing a curve in shape like that of Group 1 but at a much lower level. Finally, the capsulated streptococcus differs from all the other organisms studied in its low migration velocity at all pH values. The highest recorded

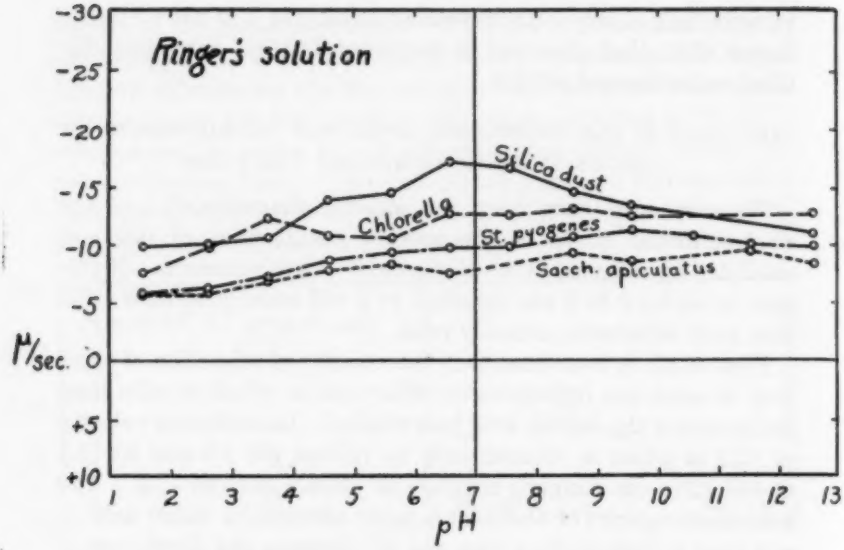
Ringer's solution
Silica dust
Chlorella
St. pyogenes
Sacch. apiculatus
μ/sec.
-30
-25
-20
-15
-10
-5
0
+5
+10
1 2 3 4 5 6 7 8 9 10 11 12 13
pH

FIG. 5

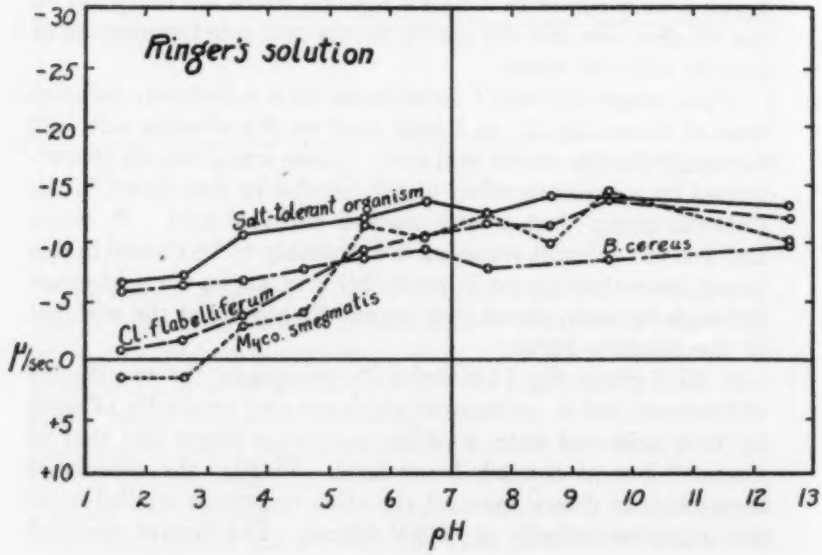
Ringer's solution
Salt-tolerant organism
B. cereus
Cl. flabelliferum
Myco. smegmatis
μ/sec.
-30
-25
-20
-15
-10
-5
0
+5
+10
1 2 3 4 5 6 7 8 9 10 11 12 13
pH

FIG. 6

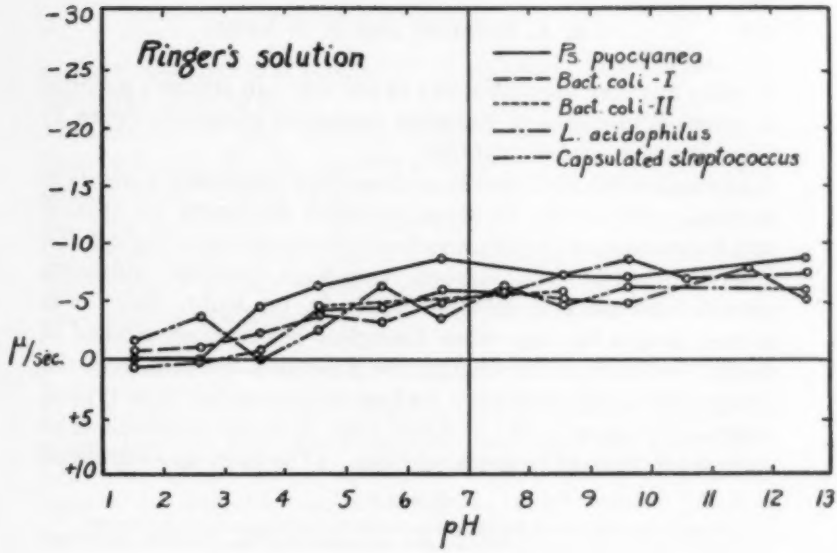
Ringer's solution
Ps. pyocyanea
Bact. coli - I
Bact. coli - II
L. acidophilus
Capsulated streptococcus
-30
-25
-20
-15
-10
-5
μ/sec. 0
+5
+10
1 2 3 4 5 6 7 8 9 10 11 12 13
pH

FIG. 7

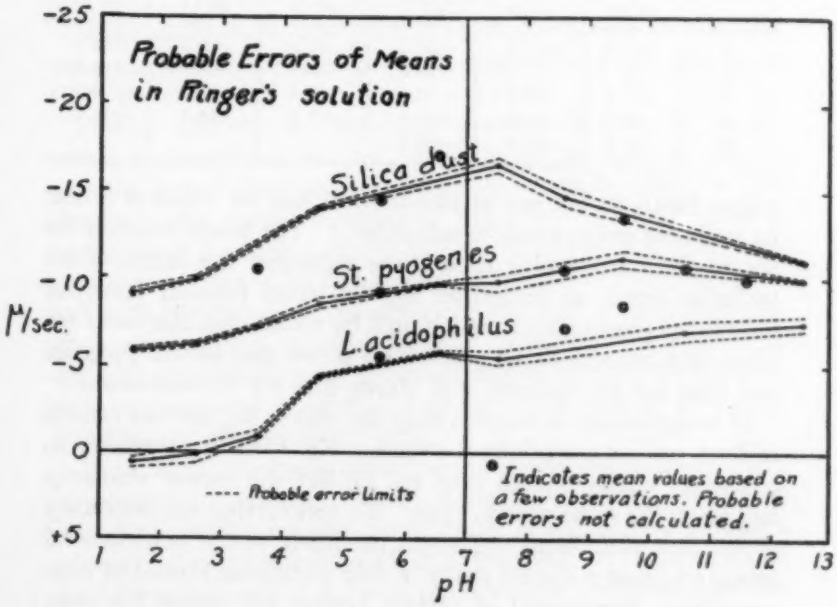
Probable Errors of Means in Ringer's solution
Silica dust
St. pyogenes
L. acidophilus
-25
-20
-15
-10
μ/sec.
-5
0
+5
---- Probable error limits
• Indicates mean values based on a few observations. Probable errors not calculated.
1 2 3 4 5 6 7 8 9 10 11 12 13
pH

FIG. 8

velocity in water was 12.9 micra at pH 8.5. In Ringer's solution its curve is like that of the other organisms plotted in figure 7, lying at a low level throughout.

In considering such results as those here presented, a question naturally arises as to the extent to which the figures are reliable and the consequent weight which may be placed upon any specific differences observed. Marked variations between duplicate observations made at different times do, no doubt, occur as is almost always the case when biological reactions are studied in detail. In order, to see whether the differences between the main groups were really significant we have computed for three typical curves (see figure 8) the probable error of the mean velocities at various pH value in Ringer's solution. (The large dots represent

TABLE 5

Migration velocities of five different strains of non-capsulated streptococci

STRAIN	WATER			RINGER'S SOLUTION		
	pH 2.5	pH 5.5	pH 9.5	pH 2.5	pH 5.5	pH 9.5
1	−2.9	−17.2	−20.4	−6.2	−9.2	−11.2
2	0	−15.7	−20.8	−0.8	−6.0	−6.4
3	−5.8	−20.5	−20.8	−5.9	−7.3	−9.1
4	−8.1	−16.2	−15.6	−7.7	−10.5	−12.2
6	−3.4	−22.9	−16.4			

values based on but one or two observations for which of course no probable errors could be calculated.) The bands between the dotted lines enveloping each curve represent the limits of the probable error, as computed by the usual formula from our individual observations; and it will be noted that the band for silica dust remains at all points well above that for *St. pyogenes* and that for *St. pyogenes* well above that for *L. acidophilus*.

It is significant to observe that the curves for the two strains of *Bact. coli* are essentially the same. No. I shows a tendency to a slightly higher charge than no. II but the curves evidently belong to the same general type. To test further the constancy of reaction of different organisms belonging to the same general group we made a special study of four additional strains of non-capsulated streptococci at certain typical pH ranges for com-

parison with our first strains of *St. pyogenes*. The results are presented in table 5.

Strain no. 1 (our primary strain) was long-chained non-hemolytic and did not produce green coloration on blood agar; strains 2 and 3 were both of the *Str. viridans* type from blood cultures; strain 4 was a non-hemolytic type from urine; strain 6 was a hemolytic strain. All of these five organisms were essentially similar in their reactions although no. 2 showed a slightly lower charge than the others.

SUMMARY OF CONCLUSIONS AND DISCUSSION OF RESULTS

It seems evident from our work, as well as from that of the other observers who have been cited, that vegetable microörganisms, like silica dust particles and collodion particles, normally bear a considerable negative charge, which causes them to migrate toward the anode in an electrical field. If the usual assumptions be made as to dielectric constant, etc., this migration velocity corresponds to a charge of about 17 millivolts.

Slight additions of acid (pH 6.5) or alkali (pH 8.5) or of neutral salts, cause an increase in velocity corresponding to a charge of 18 to 20 millivolts.

Further addition of electrolytes causes a decrease in charge, which is dependent on the concentration of cations (NaOH and NaCl producing identical effects as shown by Winslow and Fleeson). As would be expected this effect of the cations is proportional to their valency but the hydrogen ion exhibits a special power due to its high migration velocity. With hydrogen and with trivalent ions the charge on the cells is frequently entirely reversed and becomes positive. This does not occur with ordinary concentrations of univalent cations but may be noted in very extreme concentration (in alkali at pH 14.0 as shown by Winslow and Shaughnessy, 1924).

In a mixture of cations other than hydrogen the resultant effect is generally cumulative. With a mixture of hydrogen and other univalent cations on the other hand (such as acid Ringer's solution) a very interesting effect is observed. The influence of the hydrogen ion is to a considerable extent neutralized by the

ions of less potency. We are inclined to believe that in some fashion not clearly understood the sodium ions replace hydrogen ions in union with the cell or in its electrical double layer and thus produce an effect analogous to that of a buffer, although there is of course no buffer effect in the ordinary sense, since the pH value of the more effective water and the less effective salt solution is identical.

While these general effects are always manifest, distinct and significant differences may be observed between electrophoretic migration curves plotted for various types of suspended particles. Silica dust is much less markedly reduced in charge than are the vegetable cells, by either hydrogen or other cations. *Chlorella* is least affected of the organisms studied. The streptococci and *Sacch. apiculatus* are somewhat more strikingly reduced in charge while colon bacilli *Ps. pyocyanea* and *L. acidophilus* show an even greater reduction in velocity. *Myco. smegmatis* and *Cl. flabelliferum* are only moderately affected by salts (yielding a curve in the alkaline range like that of *Chlorella*) but are markedly affected by hydrogen, falling off very sharply in the acid range. *B. cereus* and the salt-tolerant organism are somewhat aberrant, showing only a moderate fall in charge with increased acidity in Ringer's solution but dropping rapidly in water in the presence of acid.

A capsulated streptococcus studied shows a marked difference from any other type in its extremely low velocity of migration. The reasons for these differences can only be conjectured. There is apparently no relation to the Gram reaction such as has been postulated by Stearn and Stearn (1925), since *Bact. coli* and *L. acidophilus* show identical results. It is perhaps significant that the yeast and the alga which were the largest organisms studied showed less effect of electrolytes than did the bacterial cells and that the capsulated organisms (perhaps on account of the chemical nature of their capsule) showed a very low velocity under all conditions. The differences observed seem in any case to be reasonably constant and significant and to indicate that the chemical and physical constitution of the cell plays at least some part in determining the effect of electrolytes upon electrophoretic charge.

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